Nutritive Value for Rats of Certain By-products of the Corn Refining Industry [1]

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ABSTRACT Experiments with growing rats were carried out to evaluate the egg replacement value and growth-promoting ability in relation to their amino acid content of by-products and a combination of by-products from wet milling of corn. In the first experiment, 8 diets containing 12.4% crude protein derived entirely from whole dried egg, corn steepwater solids, corn gluten, corn germ oilmeal, corn fine bran, zein, zein-extracted gluten, or reconstituted starch-free corn were fed to 170-g rats. Despite a superior chemical potential based on chemical score and essential amino acid index, the protein quality of corn germ oilmeal, as measured by egg replacement values, was lower than that of all of the other proteins except corn fine bran. Reconstituted starch-free corn protein was equal to corn gluten and zein-extracted gluten which were the best individual protein sources. In the second experiment protein from the same sources, excluding corn fine bran, was fed to weanling rats in diets containing 17% crude protein. Growth and efficiency of protein utilization confirmed the results of the first experiment in that corn germ oilmeal would not support growth and that reconstituted starch-free corn produced the most rapid and efficient gains of the corn proteins tested. However, growth and efficiency were appreciably poorer than when egg protein was fed.

In the wet milling of corn, which is carried out primarily to obtain starch, dextrine and oil, a number of by-products of relatively low protein quality are produced. At present these products, such as corn gluten, corn fine bran, corn coarse bran, and corn steepwater solids, are utilized chiefly in ruminant rations.

The protein of whole corn has been reported to be of relatively low quality as measured biologically or by its content of essential amino acids (1). However, there is evidence indicating that corn germ protein is superior to that found in other parts of the kernel. There is considerable variation in quality of the protein in corn germ oilmeal which is apparently dependent upon processing conditions. Most experiments indicate that it is of good quality if prepared by dry milling and solvent extraction (2, 3), but Schulz and Thomas (4) reported a low biological value for the protein in corn germ oilmeal produced by a commercial expeller process used in the wet milling of corn. Corn gluten protein is of poor quality (4, 5). Corn steepwater solids, which contain about 30% of the nitrogen in the original corn, contain a poor balance of amino acids, over 25% being present as alanine (6). Corn steepwater solids contain about 10% minerals and are a good source of B-vitamins, with the exception of thiamine. Therefore, it appears possible that a recombination of by-products resulting from the wet milling of corn might result in a product with an acceptable nutritive value for non-ruminant animals. A series of experiments have been carried out to test this possibility.

The trials reported here were carried out specifically to determine the relative growth promoting ability and egg replacement value of the protein in a number of by-products of the wet milling of corn, fed singly and combined, in relation to their chemical potential based on amino acid analyses.

EXPERIMENTAL

Experiment 1. Eighty albino rats, divided equally as to sex and weighing 170± 10 g initially, were housed in individual metabolism cages throughout this trial. Ten replicates of 8 rats each were con-

Received for publication September 6, 1966.

[1] Presented in part before the Nutrition Society of Canada, June, 1960, at Guelph, Ontario, Canada.

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TABLE 1

Proximate composition of sources of protein fed to rats in experiments 1 and 2

Protein source	Moisture	Crude protein	Ether extract	Ash	Crude fiber	N-free extract
	%	%	%	%	%	%
Dried whole egg	3.7	46.5	35.6	3.3	0.0	10.9
Corn steepwater solids	5.7	20.3	1.4	7.3	0.0	65.3
Corn gluten	5.8	59.1	2.0	1.1	0.7	31.3
Corn germ oilmeal	4.8	24.1	0.9	2.5	8.2	59.5
Corn fine bran	6.2	14.4	0.6	0.5	13.2	65.1
Zein	7.9	90.0	0.0	0.1	0.0	2.0
Zein-extracted gluten	7.4	47.4	0.6	10.9	0.2	33.5
Reconstituted starch-free corn	5.5	26.8	1.0	2.6	7.5	56.6

ducted, using all males and all females in alternative replicates.

Eight diets were fed in each replicate. The protein in each of these diets was derived from the following sources and made up the percentage of the diet indicated: whole dried egg, 26.7; corn germ oilmeal, 51.5; corn steepwater solids, 61.1; corn gluten, 21.0; zein-extracted gluten (glutelin), 26.2; zein, 13.8; reconstituted starch-free corn,[3] 46.3; and corn fine bran, 86.1.[4] The proximate composition of each protein source is shown in table 1. The protein level in each diet was adjusted to 12.4% by addition of sucrose, and the diets were made isocaloric by addition of appropriate amounts of corn oil. The diets contained 2% of a mineral-vitamin supplement.[5]

Each replicate was of 3 weeks' duration. During the first week, feed intake was determined for each rat in order to establish a maximal daily allowance of all diets that would be completely consumed in a 24-hour period. This was followed by a 4-day adjustment period, and during the last 10 days all feces and urine excreted by each rat were composited separately and frozen for subsequent analyses. The protein quality in each product was expressed as a replacement value (RV) for the protein of dried whole egg according to the method of Murlin et al. (7). The apparent digestibilities of dry matter and crude protein were calculated by the index method described by Schürch et al. (8).

Experiment 2. This experiment consisted of a comparative growth and digestibility trial using 70 weanling rats divided equally as to sex. They ranged in initial age from 28 to 35 days, and were divided at random into 7 lots of 5 males and 5 females, each rat being housed individually.

The protein in each of the 7 diets was derived from the same lots of egg and corn by-poducts used in experiment 1. The percentage of each incorporated in the diets were: dried whole egg, 36.6; corn steepwater solids, 83.7; corn gluten, 28.8; corn germ oilmeal, 70.5; zein, 18.9; zein-extracted gluten, 35.9; and reconstituted starch-free corn, 63.4. Corn fine bran was not included as it could not be used at a level high enough to provide the crude protein level of 17% used in the other diets. As in experiment 1, all diets were made isocaloric by the addition of corn oil, and each contained 2% added minerals and vitamins.[6]

All diets were fed ad libitum for 4 weeks. During the last 10 days, feces samples of the 10 rats fed each diet were composited daily. Weekly records of weight change and feed intake were kept. Protein efficiency ratios[7] were calculated based on body weight gains and feed consumption.

[3] Reconstituted according to mill-run proportions of the following by-products: (in per cent) corn steepwater solids, 21.0; corn gluten, 22.6; corn fine bran, 9.0; corn germ oilmeal, 25.8; and corn coarse bran, 21.6.

[4] These corn by-products were supplied by the Corn Products Company, Argo, Illinois, and were produced by wet-milling procedures, the oil being removed by an expeller.

[5] The composition of the mineral-vitamin mix was: (in per cent) Ca biphosphate (monobasic), 7.80; Ca lactate, 18.80; ferric citrate, 1.70; magnesium sulphate, 7.88; potassium phosphate (dibasic), 13.80; sodium biphosphate (monobasic), 5.02; sodium chloride, 2.50; chromic oxide, 25.0; vitamin A (250,000 IU/g), 0.39; vitamin D_3, (1,654 IU/g), 6.62; α-tocopherol, 0.55; thiamine·HCl, 0.11; riboflavin, 0.11; niacin, 0.49; Ca pantothenate, 0.33; pyridoxine·HCl, 0.11; choline chloride, 8.21; inositol, 0.55; folic acid, 0.01; and biotin, 0.0025.

[6] See footnote 5.

[7] Gain per gram of protein consumed.

RESULTS AND DISCUSSION

Experiment 1. The egg replacement values (RV's) of the protein of zein-extracted gluten, corn gluten or corn steepwater solids were not statistically different ($P < 0.05$) from that of reconstituted starch-free corn (table 2). The RV of gluten protein was not improved by the removal of zein. This was apparently due to a significant ($P < 0.05$) reduction in digestibility of zein-extracted gluten protein, presumably as a result of the extraction process. Moreover, the percentage retention of digested nitrogen was not improved by removal of zein from corn gluten.

The RV's of zein, corn germ oilmeal and corn fine bran proteins were significantly lower than that of reconstituted corn protein. However, the very low RV of corn germ oilmeal was somewhat unexpected and probably resulted from conditions peculiar to the wet-milling procedures. A number of workers (2, 9) have reported that corn germ oilmeal protein is equal in quality to soybean protein, and Block and Bolling (10) unexpectedly obtained equal protein efficiency ratios for milk and corn germ oilmeal proteins. Beeson et al. (11) obtained a relatively good protein efficiency ratio of 2.01 for corn germ oilmeal, as compared with 2.16 for wheat germ and 2.50 for egg protein. However, Jones and Widness (12) reported corn germ protein to produce less efficient gains than casein, soybean oilmeal, wheat germ or whole egg protein.

The unexpected low quality of corn germ oilmeal protein was traceable, to a considerable degree, to its relatively low digestibility of 70%. This value was significantly lower ($P < 0.05$) than the protein digestibility of corn steepwater solids, corn gluten, zein-extracted gluten, and reconstituted starch-free corn; and was of the same order as that of corn fine bran. Only zein had a significantly lower value ($P < 0.05$) than that of corn germ oilmeal. The retention of digested nitrogen from corn germ oilmeal and from zein was similar.

Chemical scores and the essential amino acid indexes were calculated, as described by Mitchell and Block (13) and Oser (1), respectively, from amino acid analyses of the 7 proteins.[8] The results are presented in table 3.

Both methods ranked the 7 corn by-products in the same order. Corn germ oilmeal exhibited the highest chemical potential and was followed closely by zein-extracted gluten. Reconstituted starch-free corn, corn gluten, corn steepwater solids and corn fine bran followed in decreasing quality, but within a fairly narrow range. As expected, zein displayed the lowest chemical potential.

It is of importance that the chemical potential of corn germ oilmeal was not fully realized in terms of RV. There is some evidence to support the hypothesis that some phase of the oil extraction was responsible for the low digestibility which resulted in low RV. Schiller (14) reported that the high biological value of corn germ was reduced during oil extraction; and

[8] The microbiological assays were carried out by the Wisconsin Alumni Research Foundation, Madison, Wisconsin.

TABLE 2

Results of the nitrogen balance and digestibility trial using 170-g rats [1]

Protein source	Egg replacement value	Retention of digested nitrogen	Dry matter digestion coefficient	Crude protein digestion coefficient
		%	%	%
Dried whole egg	—	17^{a}	97^{a}	91^{a}
Zein-extracted gluten	91^{a}	8^{ab}	91^{c}	77^{b}
Corn gluten	90^{ab}	7^{ab}	96^{ab}	91^{a}
Reconstituted starch-free corn	86^{ab}	2^{b}	82^{d}	79^{b}
Corn steepwater solids	81^{abc}	-4^{b}	95^{b}	89^{a}
Zein	74^{cd}	-21^{c}	92^{c}	53^{d}
Corn germ oilmeal	72^{cd}	-17^{c}	80^{d}	70^{c}
Corn fine bran	56^{c}	-44^{d}	59^{e}	67^{c}

[1] Values followed by the same superscript letter do not differ ($P < 0.05$).

Schulz and Thomas (4) observed commercially solvent-extracted corn germ to have a biological value of 83, and of 66 if prepared by a commercial expeller process similar to that used for preparation of the meal tested in this experiment.

Experiment 2. Average daily gain, average daily feed intake and protein efficiency ratios from the rat growth experiment are shown in table 4.

None of the corn by-products promoted growth at a rate equivalent to that observed when egg protein was fed ($P < 0.05$). Feeding corn steepwater produced small gains or weight losses, but gains in groups fed zein-extracted gluten and starch-free corn were significantly greater ($P < 0.05$) than for the other corn protein sources.

Feed consumption by groups fed egg, zein-extracted gluten, and starch-free corn did not differ significantly ($P < 0.05$), but were greater ($P < 0.05$) than for the other protein sources. Protein efficiency ratios were calculated for the 4 groups which gained weight. The highest efficiency was observed when egg protein was fed, followed in descending order by starch-free corn, zein-extracted gluten and corn gluten, with the value for each diet differing significantly ($P < 0.05$) from those of the other three diets.

Although growth rates and protein efficiency ratios were low in relation to egg, the protein of starch-free corn was more efficiently utilized ($P < 0.05$) than that of any single corn protein source, and also the zein-extracted gluten protein was used more efficiently than when the zein was not extracted. This improved efficiency undoubtedly resulted more from a change in amino acid balance than from the removal of poorly digested zein (table 5), because the digestibility of the remaining glutelin protein appeared to have been markedly reduced after the extraction of zein.

The rats fed the corn germ oilmeal diet developed a rough hair coat early in the

TABLE 3

Chemical potential of the protein of corn by-products according to their essential amino acid content [1]

Corn by-product	Chemical score method		Essential amino acid index (EAAI) method
	Chemical score	Limiting amino acid	EAAI
Corn germ oilmeal	51	methionine	89
Zein-extracted gluten	48	lysine	88
Reconstituted starch-free corn	43	tryptophan	75
Corn gluten	33	tryptophan	70
Corn steepwater solids	30	tryptophan	68
Corn fine bran	30	lysine	61
Zein	1	lysine	39

[1] Microbiological assays carried out by the Wisconsin Alumni Research Foundation.

TABLE 4

Results of the growth trial using weanling rats [1]

Source of dietary protein	Avg daily gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
Dried whole egg	3.62[a]	8.8[a]	2.42[a]
Reconstituted starch-free corn	2.28[b]	9.6[a]	1.38[b]
Zein-extracted gluten	1.82[b]	8.8[a]	1.21[c]
Corn gluten	0.45[c]	5.5[b]	0.45[d]
Corn germ oilmeal	0.00[c]	4.6[bc]	—
Corn steepwater solids	−0.16[d]	4.2[bc]	—
Zein	−0.67[e]	3.4[c]	—

[1] Values followed by the same superscript letter do not differ ($P < 0.05$).

experiment, and 40% mortality was observed. These rats probably did not die of starvation as the stomach and intestines contained food material. Draper (15) has reported similar mortality rates with both rats and chicks fed diets containing 30% or more corn germ oilmeal in the diet. There is no evidence indicating that whole corn contains a toxin. However, Borrow et al. (16) have reported a growth-retarding factor in corn bran fed to mice, and Lipke and Fraenkel (17) observed a growth inhibition by corn germ fed to the meal worm, *Tenebrio molitor*.

Differences in dry matter digestibility between diets (table 5) were largely a reflection of variation in crude fiber content and in digestibility of crude protein or nitrogen-free extract. Ether extract digestibility was high in all cases.

A comparison of dry matter and crude protein digestibilities between the 2 experiments indicates that the two age groups digested the diets to similar extents. Zein was an exception in that the older rats used in experiment 1 digested this protein to a greater extent. The low digestibilities by the older rats in experiment 1 for corn germ oilmeal and zein-extracted gluten proteins (70 and 77%, respectively) were confirmed in experiment 2 (71 and 76%, respectively).

In keeping with the observations of Bolton (18), the digestibility of the nitrogen-free extract of corn germ oilmeal was somewhat low. This is probably due to the poor availability of nitrogen-free extract in the fiber portion, because the other fiber-containing by-product, starch-free corn, also had a low nitrogen-free extract digestibility.

The extent to which the essential amino acid requirements of weanling rats were met by the various diets is illustrated in table 6. Only the diet containing whole egg protein provided each essential amino acid in the minimum required amounts. The apparent deficiency of methionine (0.53% supplied and 0.60% required) would be met by cystine. Each of the diets containing corn by-products exhibited essential amino acid deficiencies, the most frequent deficiencies being lysine, methionine, and tryptophan. The inadequacy of Mitchell's chemical score based on egg protein in determining the limiting amino acid can be observed. In table 3, tryptophan is shown to be the amino acid limiting growth in reconstituted starch-free corn and in corn gluten. However, based on estimated amino acid requirements (19), lysine is the growth-limiting amino acid in both reconstituted starch-free corn, and corn gluten.

These 2 experiments emphasize that although a protein such as that in corn germ oilmeal may possess a high chemical potential in terms of its content and balance of essential amino acids, this potential may be biologically meaningless if digestion and absorption are impaired.

TABLE 5

Results of the digestibility trial using weanling rats

Source of dietary protein	Apparent digestion coefficients				
	Dry matter	Crude protein	Ether extract	Crude fiber [1]	N-free extract
	%	%	%	%	%
Dried whole egg	97	93	91	—	99
Reconstituted starch-free corn	75	78	94	28	72
Zein-extracted gluten	88	76	97	—	94
Corn gluten	95	93	96	—	97
Corn germ oilmeal	76	71	97	39	75
Corn steepwater solids	94	88	97	—	97
Zein	87	39	97	—	98

[1] Blank values indicate negligible levels of crude fiber in the diet.

TABLE 6

Essential amino acid (EAA) content of diets fed in experiment 2 and corresponding requirement of the weanling rat

Amino acid	EAA composition of diets							EAA requirements of weanling rat (19)
	Dried whole egg	Corn steep-water solids	Corn gluten	Corn germ oilmeal	Zein	Zein-extracted gluten	Reconstituted starch-free corn	
	% of diet	*% of diet*	*% of diet*	*% of diet*	*% of diet*	*% of diet*	*% of diet*	*% of diet*
Arginine	1.00	0.48	0.58	0.98	0.32	0.71	0.65	0.20
Histidine	0.42	0.53	0.42	0.67	0.28 [1]	0.49	0.52	0.30
Isoleucine	1.01	0.57	0.79	0.72	0.87	0.74	0.71	0.50
Leucine	1.62	1.46	1.01	1.43	3.87	2.58	1.23	0.80
Lysine	1.13	0.64 [1]	0.37 [1]	0.85 [1]	0.01 [1]	0.54 [1]	0.54 [1]	0.90
Methionine	0.53	0.38 [1]	0.44 [1]	0.27 [1]	0.31 [1]	0.51	0.36 [1]	0.60 [2]
Phenylalanine	1.06	0.64 [1]	1.23	0.79 [1]	1.39	1.16	0.96	0.90
Threonine	0.72	0.65	0.69	0.67	0.44 [1]	0.64	0.67	0.50
Tryptophan	0.23	0.07 [1]	0.08	0.17	0.01 [1]	0.12 [1]	0.10 [1]	0.15
Valine	1.00	1.05	1.01	1.57	0.88	1.21	1.15	0.70

[1] Below requirement.
[2] One-third to one-half may be supplied as L-cystine (19). Cystine levels in these proteins were not determined.

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Metabolism of Hydroxylysine by Rats [1]

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ABSTRACT DL- and DL-allo-5-hydroxylysine-6-^{14}C and L-5-hydroxlysine-^{3}H were administered intraperitoneally to intact rats and the degradative products were trapped by the metabolite overloading technique. Hydroxylysine was readily oxidized; approximately 40% of the ^{14}C was expired as $^{14}CO_2$ in 24 hours. Urinary 5- hydroxypipecolate, α-ketoadipate, glutarate and crotonate were identified as labeled degradation products isolated from the urine. The specific activities of glutamate, aspartate and alanine isolated from the carcass protein were too low to permit the determination of the labeling pattern. This low activity and the rapid release of $^{14}CO_2$ suggests that $C_{(6)}$ of hydroxylysine reaches these amino acids via $^{14}CO_2$. The degradation of hydroxylysine appears to proceed by reactions parallel to those which degrade lysine with the site of dehydroxylation as yet uncertain.

Hydroxylysine (2,6-diamino-5-hydroxycaproic acid), a component of collagen, has been studied metabolically to only a limited extent although it was isolated from gelatin many years ago (1). The hydroxylysine of collagen arises from dietary lysine and not from exogenous hydroxylysine (2). In bacteria, interchangeability, but not interconversion, of lysine and hydroxylysine has been observed.[4]

It might be expected then that the slow turnover of collagen in animals would require degradation of the resulting hydroxylysine which has no other known function in animals. The degradation of lysine has been investigated extensively. The most widely accepted pathway involves pipecolate, α-aminoadipate and glutaryl coenzyme A (3–5). If a similar pathway serves for degrading hydroxylysine, the corresponding hydroxy compounds, hydroxypipecolate, 2-amino-5-hydroxyadipate and hydroxyglutarate might be expected to be involved. Evidence for the participation of 5-hydroxy-L-pipecolate was obtained by Lindstedt and Lindstedt (6) using rat kidney and liver homogenates to degrade DL-hydroxylysine. A later report from the same laboratory (7) refers to evidence that 2-amino-5-hydroxyadipate is formed from hydroxylysine, possibly enroute to the corresponding keto-derivative, 2-hydroxyglutarate and 2-ketoglutarate. The present communication confirms the involvement of 5-hydroxypipecolate, but suggests an early loss of the hydroxyl group leading to α-ketoadipate, glutarate and crotonate as products detectable by metabolite overloading experiments in the intact rat.

EXPERIMENTAL PROCEDURE

Chemicals. L-5-Hydroxylysine was isolated from gelatin hydrolysate by ion exchange chromatography (8). It was shown to contain no more than 1 to 5% of D-allohydroxylysine [5] and was labeled with tritium by the Wilzbach procedure (9). This labeled hydroxylysine is the same sample described previously (10). It has been estimated that no more than 5% racemization might occur during the tritium labeling.[6]

5-Hydroxy-L-pipecolic acid was isolated from dates by the procedure of Witkop and Foltz (11). A mixture of DL- and DL-allo-5-hydroxylysine-6-^{14}C was obtained from New England Nuclear Corporation;

Received for publication September 21, 1966.

[1] This work was supported by a grant from the National Institutes of Health (5-R01-AM07984-04). Scientific Journal Series no. 5890 Agricultural Experiment Station, University of Minnesota, St. Paul, Minnesota.

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[4] Leach, F. R., W. G. Smith and C. M. Tsung 1961 Metabolism of hydroxylysine by *Streptococcus faecalis*. Federation Proc., 20: 4 (abstract).

[5] Dr. Paul Hamilton of Alfred I. duPont Institute of the Nemours Foundation, Wilmington, Delaware, kindly examined this product by the ion exchange procedure.

[6] Personal communication from Dr. W. Richard Waterfield, Radiochemical Centre, Amersham, England.

this product will hereafter be referred to as the "mixed isomers." Other compounds used were obtained commercially. α-Ketoadipic acid and glutaric acid were recrystallized and melted at 128° and 98°, respectively.

Animal experiments. Tritiated 5-hydroxy-L-lysine was used in 2 experiments; in all other experiments the mixed isomers, labeled with ^{14}C in the 6-position were used. Male rats weighing 200 to 290 g were injected, intraperitoneally, with 10 to 20 mg (10–200 μCi) of labeled compound followed immediately by an intraperitoneal injection of 1 to 3 mmoles of a suspected metabolic product at pH 7.4. The rats were placed in glass metabolism cages for 24 hours while CO_2 was collected in 1 N NaOH by sweeping the cage with CO_2-free air. The compounds of interest were isolated from the urine collected during the 24-hour period.

In one experiment, in which the mixed isomers of hydroxylysine-^{14}C were used, carcass protein was isolated (12), hydrolyzed, treated with charcoal and the amino acids were isolated by ion exchange chromatography (13). The specific activity of the nonessential amino acids was determined by established techniques (12) to estimate the incorporation of carbon from $C_{(6)}$ of hydroxylysine.

ISOLATION PROCEDURES

Urine collected from rats given overloading doses of glutarate, glutaconate, or α-ketoadipate, was taken to dryness by lyophilization after neutralization with NaOH. The dry residue was dissolved in 0.5 N H_2SO_4 and chromatographed on silica gel columns along with authentic carrier acids by a modification of the procedure of Marvel and Rands (14).

5-Hydroxypipecolate was isolated from urine using an Amberlite IR-120 (H^+) column (30 × 2.6 cm) with 1.5 N HCl as the developing solvent (11). The carrier 5-hydroxypipecolate was located in the effluent by ninhydrin color and the concentrations were determined by the method of Piez et al. (15) for cyclic amino acids. Pipecolate was isolated by the method of Schweet et al. (16).

Liquid scintillation counting was used for both ^{14}C and ^{3}H. The scintillation fluid consisted of 4.0 g of 2,5-diphenyloxazole, 0.2 g 1,4-di [2-(*t*-phenyloxazolyl)] benzene per liter of 40% absolute ethanol and 60% toluene.

RESULTS

The mixed isomers of hydroxylysine were readily oxidized by the rat. The $^{14}CO_2$ was released at a rate shown in figure 1 except in the experiment in which 5-hydroxypipecolate was used as the overloading metabolite, when the $^{14}CO_2$ release was reduced 60 to 70%.

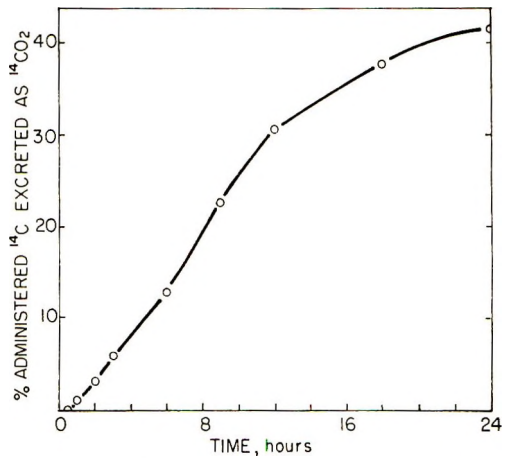

Fig. 1 Rate of excretion of $^{14}CO_2$ after administration of 18 mg (20 μCi) of DL- and DL-allo-5-hydroxylysine-6-^{14}C and 2 μmoles of glutaconic acid, as separate injections, into a 225-g male rat.

The information obtained from the labeling of nonessential amino acids of the carcass proteins was not very helpful. The labeling pattern, which might have been useful in identifying the small fragments arising from $C_{(6)}$ of hydroxylysine, could not be determined since the specific activity of aspartate (2.2 mμCi/mmole) glutamate (1.6 mμCi/mmole) and alanine (1.7 mμCi/mmole) were too low to permit the accurate determination of the labeling pattern when 20 μCi of the mixed isomers were injected. Much higher specific activities would be expected (17) in each of these amino acids if the labeled carbon were metabolized by a pathway which leads directly into the tricarboxylic acid cycle as suggested by Lindahl et al. (7). The rapid release of $^{14}CO_2$ and the

low specific activity of these amino acids suggest that the amino acids become labeled by fixation of $^{14}CO_2$ and not by the more efficient labeling available through acetate, pyruvate etc.

The direct release of $C_{(6)}$ of lysine as CO_2 (18) suggested experiments in which lysine degradation products and the compounds expected to arise from corresponding transformation of hydroxylysine were tested by the metabolite-overloading technique. Glutarate was labeled under these conditions as evidenced by the correspondence between ^3H and titration elution patterns (fig. 2) from a silica gel column separation of the products from urine following overloading with glutarate. The urinary glutarate contained 6.9% of the ^3H present in the urine.

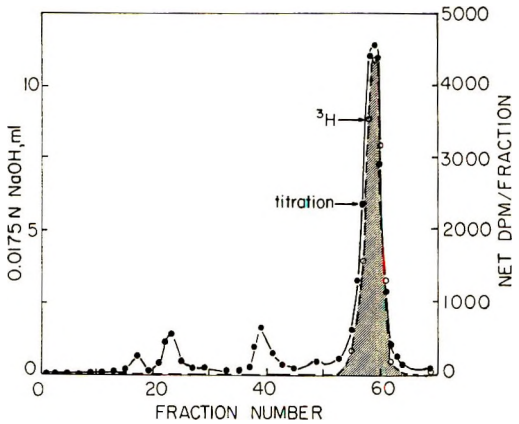

Fig. 2 Coincidence of titration values (●) and radioactivity (○) of 5-ml fractions from the silicic acid chromatographic analysis of a 24-hour urine collection from a 200-g rat that received 11.1 mg (11.1 μCi) of tritiated L-5-hydroxylysine and 300 mg of glutaric acid at zero time; 20 mg of carrier glutaric acid were added to the urine residue prior to chromatography.

If α-hydroxyglutarate were an intermediate in hydroxylysine degradation, as suggested by Lindahl et al. (7), glutaconate might arise by dehydration. Glutaconate might also arise through dehydration of 2-keto-5-hydroxyadipic acid to form α-hydroxymuconate which could be oxidatively decarboxylated to glutaconyl CoA. To test these possibilities, rats were injected with ^{14}C-hydroxylysine and large quantities of glutaconate. With the procedure described above the results suggested that glutaconate was labeled, but the peak effluent volume of glutaconate is very close to that for glutarate and preliminary experiments suggested that glutarate, not glutaconate, was the labeled product. This was confirmed by repeating the experiment, then isolating the mixture of glutarate and glutaconate by silica gel chromatography after adding carrier glutarate (0.11 mmole) and glutaconate (0.23 mmole). The radioactive mixture of the 2 acids was subjected to ozonolysis in ethanol. The ozonolysis product was oxidatively decomposed by the method of Bailey (19) and the products were separated on a silicic acid column. No ^{14}C was found in the fractions containing malonate and oxalate, the ozonolysis products of glutaconate, but the ^{14}C was in the glutarate containing fractions from the column. After adding 50 mg of additional carrier glutarate to these fractions glutaric acid was isolated and recrystallized 3 times without change in the specific activity.

These results establish glutarate as a metabolite of hydroxylysine in which $C_{(6)}$ is retained and suggest that $C_{(1)}$ is lost as CO_2. The substrate for this decarboxylation is likely to be α-ketoadipate and the product should be glutaryl-coenzyme A labeled in the free carboxyl group. The next reaction would be the formation of crotonyl-CoA and $^{14}CO_2$. This would account for the rapid formation of $^{14}CO_2$ and the lack of extensive labeling of amino acids from $C_{(6)}$. The lack of labeling in glutaconate does not eliminate it as an intermediate since negative results cannot be interpreted (20).

Because of the unexpected observation that glutaconic acid overloading forced the excretion of glutarate, a rat was injected with the mixed isomers of hydroxylysine-6-^{14}C, without overloading with any compound. Eighteen milligrams of glutarate were added to the urine and examination of the elution pattern obtained from silica gel chromatography showed coincidence of radioactivity and the titration of carrier glutarate. In addition a large radioactive peak believed to be crotonate was observed. The peak tubes were pooled, 52 mg of crotonate added and the product was sublimed to constant specific activity (table 1). These results show that $C_{(6)}$ of hy-

TABLE 1

Identification of crotonic acid in rat urine by the carrier technique [1]

Sublimation	
	dpm/mg
First	640
Second	530
Third	505

[1] Sublimation of crotonic acid from a 24-hour collection of urine from a 290-g rat that had been administered 11.5 mg (20 μCi) DL- and DL-allo-5-hydroxylysine-6-^{14}C.

droxylysine gives rise to ^{14}C-glutarate and crotonate in the urine even when the rat was not overloaded with either compound.

α-Ketoadipate, a metabolic precursor of glutaryl-CoA in lysine degradation (21), was found to be a metabolic product of the mixed isomers of hydroxylysine-^{14}C (fig. 3). The peak area represents 2% of the urinary ^{14}C. One hundred milligrams of recrystalized α-ketoadipic acid were added to the combined radioactive fractions and crystallized to constant specific activity from ethyl acetate, thus establishing the identity of the radioactive product isolated.

α-Ketoadipate was also found to be a metabolite of hydroxylysine by its becoming labeled from the naturally occurring isomer of hydroxylysine labeled with tritium. Sixty-one milligrams of recrystallized α-ketoadipate were added to the urine which was then chromatographed on silicic acid. The fractions containing ^{3}H were then recrystallized to constant specific activity (table 2).

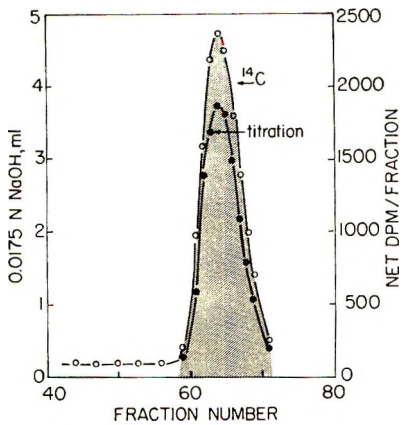

Fig. 3 Coincidence of α-ketoadipic acid titration value (●) and radioactivity content (○) of 5.2 ml fractions from the silicic acid chromatographic analysis of a 24-hour urine collection from a 225-g rat administered 20 mg (20 μCi) DL- and DL-allo-5-hydroxylysine-6-^{14}C and 315 mg of α-ketoadipic acid. This represents rechromatography of 50% of an identically separated peak to which 20 mg of carrier α-ketoadipic acid were added prior to evaporation to dryness.

TABLE 2

Identification of α-ketoadipic acid and glutaric acid in rat urine by the carrier technique [1]

Crystallization	
	dpm/mg
α-ketoadipic acid	
First	6960
Second	6430
Third	6230
Glutaric acid	
First	2840
Second	3460
Third	3460

[1] Crystallization of α-ketoadipic acid and glutaric acid isolated from a 24-hour urine collection which was obtained from a 275-g rat that had been administered 10 mg (200 μCi) of tritiated L-5-hydroxylysine and 180 mg of α-ketoadipic acid in 5 equal portions one hour apart.

5-Hydroxypipecolic acid was reported to be a product of DL-hydroxylysine-6-^{14}C formed by rat kidney or liver homogenates (6) presumably by reactions which parallel those which lead to pipecolic acid, a urinary metabolite of lysine in the rat (22). Overloading experiments were performed with both pipecolate and 5-hydroxypipecolate using ^{14}C-hydroxylysine, since if both were labeled the location of dehydroxylation in the pathway would be fixed and the route of α-ketoadipate and glutarate formation would be established.

Ion exchange chromatography of the urine of the rat overloaded with hydroxypipecolate gave an elution pattern for radioactivity which coincided closely with that observed for the ninhydrin color reaction of the carrier 5-hydroxypipecolate (fig. 4). Paper chromatography of the material isolated from the major peak, shown in figure 4, resulted in the same R_f values for the ninhydrin-positive material, the radioactivity, and for authentic 5-hydroxypipecolate in the following solvent systems: phenol:water; tertiary butanol: formic acid:water (70:15:15); 1-butanol: acetic acid:water (60:15:25) and ethanol: water: ammonia (0.88 M) (90:5:5). Radioactivity was found only in the area

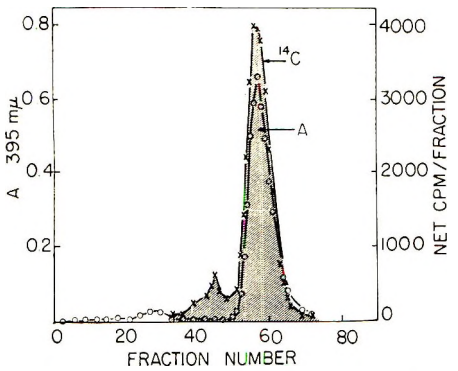

Fig. 4 Coincidence of 5-hydroxypipecolic acid ninhydrin values (○) and radioactivity (×) from Amberlite IR-120 (H^+) chromatography (see Experimental Procedure) of a 24-hour urine collection from a 220-g rat administered 30 mg (15 μCi) of DL- and DL-allo-5-hydroxylysine-6-^{14}C and 1.5 mmoles of 5-hydroxypipecolic acid. These data represent the results of rechromatography of the 5-hydroxypipecolate after adding 20 mg of additional carrier.

occupied by hydroxypipecolate. This observation confirms the report of Linstedt and Lindstedt (6) that 5-hydroxypipecolate is a product of hydroxylysine.

In a similar experiment involving overloading with pipecolate no ^{14}C was found in the pipecolate isolated. This observation does not exclude pipecolate as a product of hyroxylysine, but in view of experiments which establish this compound as a urinary excretion product of lysine and in view of the hydroxypipecolate results described above it appears not to be a product of hydroxylysine.

DISCUSSION

The rate and extent of release of $C_{(6)}$ of hydroxylysine as $^{14}CO_2$ (fig. 1) is comparable to that observed for lysine-6-^{14}C (18) and glutaric acid-1,5-^{14}C (23). The extensive degradation to CO_2 (40%) suggests that at least two of the four isomers are readily metabolized by the rat. The $^{14}CO_2$ expired progressed at a nearly linear rate for the first 12 hours of the experiment then gradually subsided. This continued $^{14}CO_2$ release for 12 hours following the administration of a single dose of a radioactive compound, contrasts sharply with the behavior of the amino acids found in proteins; in the latter case the $^{14}CO_2$ release rate is usually very low after 3 to 6 hours. Thus, the mixed isomers of hydroxylysine are metabolized promptly, but the lack of competing removal for protein synthesis results in slower dissipation of the substrate.

The low rate of utilization of $C_{(6)}$ of hydroxylysine through the tricarboxylic acid cycle is shown by the extremely low specific activity of carcass amino acids. Any substance whose degradation leads to compounds which enter the tricarboxylic acid cycle, such as labeled acetate or more distant compounds such as labeled glycerol, give rise to protein glutamate with very high specific activity (17). The low specific activity of the amino acids isolated and the high rate of release of $^{14}CO_2$ suggest that $C_{(6)}$ of hydroxylysine is released largely as CO_2 (12) and is not entering the tricarboxylic acid cycle directly as α-ketoglutarate (fig. 5) with retention of $C_{(6)}$ as has been suggested (7). The specific activity of aspartate was somewhat higher than that of glutamate again suggesting $^{14}CO_2$ fixation as the route of amino acid labeling.

The $^{14}CO_2$ and amino acid labeling results are in keeping with the view that glutaryl CoA is an intermediate in hydroxylysine degradation. Presumably $C_{(5)}$ of glutaryl CoA would arise from $C_{(6)}$ of hydroxylysine and would be lost as CO_2 in the formation of acetoacetyl CoA (20) (fig. 5). The labeling of crotonate observed in these studies requires some discussion in view of the recent report of Meghal et al. (5) that approximately 90% of the metabolism of $C_{(6)}$ of lysine to CO_2 occurs through loss of CO_2 from glutaryl-CoA in forming crotonyl-CoA. Although comparable data with hydroxylysine-6-^{14}C could not be obtained, it can be assumed that the equilibration of glutaryl-CoA with glutarate should be similar to that observed in the lysine experiments (5). The labeling observed in crotonate (crotonyl-CoA) could result from the small percentage of the crotonyl-CoA arising from glutaryl-CoA which becomes labeled in $C_{(1)}$ by randomization of ^{14}C in the 2 carboxyl groups of free glutarate (fig. 5). Overloading experiments may not differentiate between carboxylic acids and their CoA derivatives since the isolation of acids from the urine involves alkaline treatment which would hydrolyze the CoA esters.

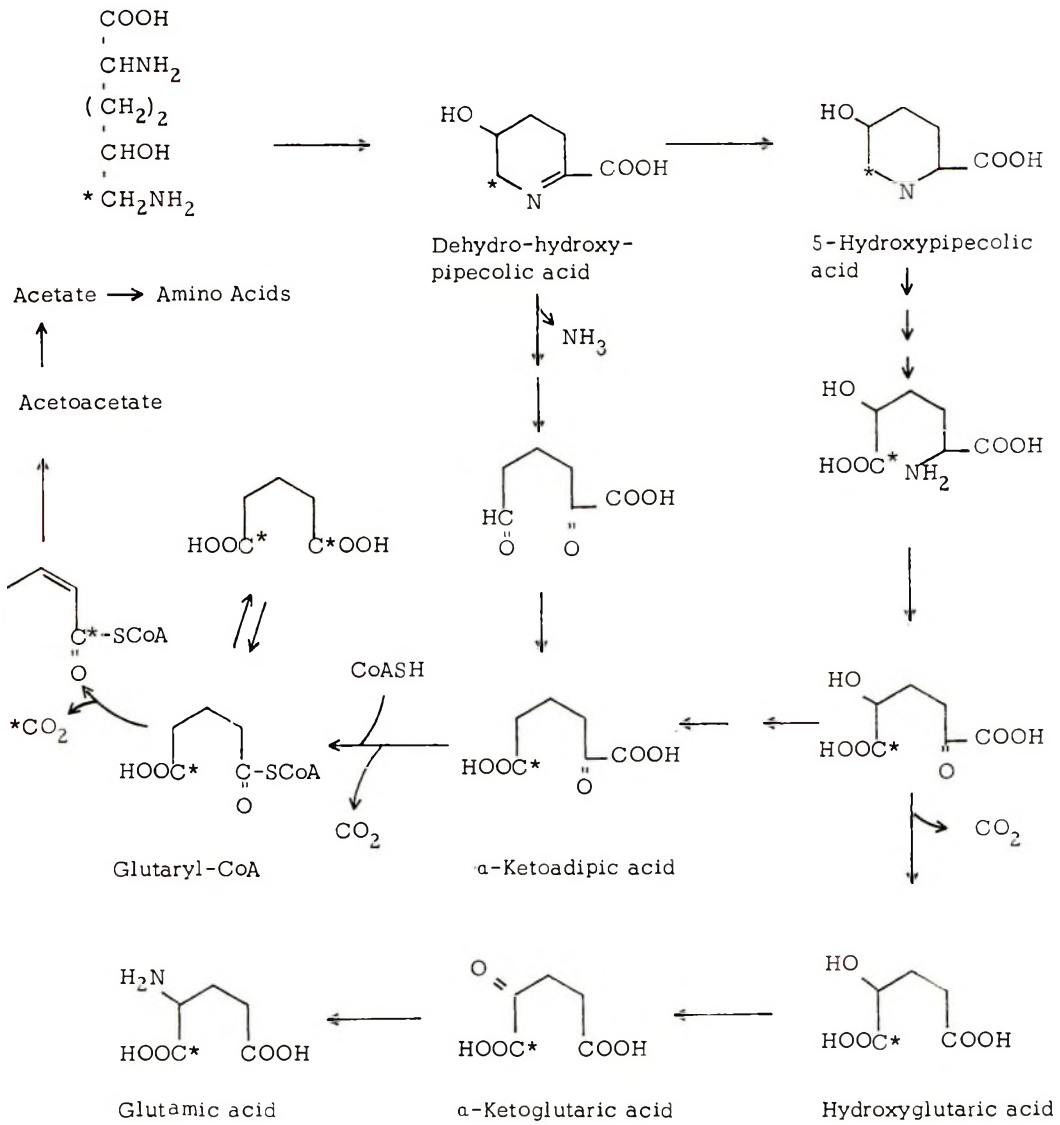

Fig. 5 Possible routes of hydroxylysine degradation.

The identification of glutarate and α-ketoadipate as products of the degradation of the naturally occurring isomer of hydroxylysine could be questioned in the ^{14}C experiments. However, the experiment with hydroxylysine-^{3}H establish these compounds as products of the natural L-isomer and support the involvement of hydroxypipecolate and crotonate as well. Further experiments with each of the 4 isomers labeled with ^{14}C are needed.

The glutaconate overloading experiment was conducted to test one possible means of obtaining glutarate from hydroxylysine, that is, dehydration of α-hydroxyglutarate to give glutaconate followed by reduction to glutarate. This negative result and the positive result with α-ketoadipate suggest that the loss of the hydroxyl group occurs earlier, giving α-ketoadipate and subsequent products in common with the lysine degradative pathway. 5-Hydroxypipeco-

late labeling confirmed observations made with tissue homogenates. The failure to observe labeled pipecolate in these studies suggests strongly that the lysine and hydroxylysine pathways do not converge at this point.

Lindahl et al. (7) refer to unpublished experiments which show that 5-hydroxylysine is converted to 2-amino-5-hydroxyadipic acid (AHA). They then showed that AHA-6-^{14}C is converted to α-hydroxyglutarate by liver homogenates. The expected 2-keto-5-hydroxyadipate was also labeled. The latter could give rise to α-ketoadipate, but the involvement of α-hydroxyglutarate appears doubtful in view of the amino acid labeling data and the labeling of α-ketoadipate and glutarate reported here.

In earlier studies with lysine degradation, it was concluded that α-hydroxyglutarate and α-ketoglutarate were direct products of L-lysine-6-^{14}C and glutarate-1,5-^{14}C degradation (24). These conclusions were based on relative specific activities of the isolated products. It was later shown that glutarate-3-^{14}C gave rise to glutamate labeled almost exclusively in $C_{(1)}$ and $C_{(5)}$ suggesting that glutarate gets to glutamate via acetate (25). Apparently α-hydroxyglutarate was labeled only because of its equilibration with the labeled α-ketoglutarate of the tricarboxylic acid cycle.

The same line of reasoning can explain the labeling of α-hydroxyglutarate observed by Lindahl et al. (7). The isotope data reported here appear to exclude hydroxylglutarate as a direct intermediate in hydroxylysine degradation. Hydroxypipecolate may be an intermediate or only in equilibrium with a true intermediate. The removal of the hydroxyl group may occur by dehydration of 2-keto-5-hydroxyadipate or at some earlier step or possibly by the involvement of the loss of NH_3 and dismutation of Δ^1-dehydrohydroxypipecolic acid to α-ketoadipic semialdehyde in a manner analygous to hydroxyproline metabolism in *Pseudomonas* (26) followed by the oxidation to α-ketoadipate (fig. 5).

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Long-term Studies on the Hypolipemic Effect of Dietary Calcium in Mature Male Rats Fed Cocoa Butter [1]

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ABSTRACT To determine whether the hypolipemic action of elevated dietary calcium previously noted in a 3-week experiment was a transient or a persistent effect, 32 mature male Holtzman-strain albino rats were divided into 5 groups, one being fed commercial rat pellets with 5% fat and 1.15% calcium, and the others a corn-soya ration containing 18% added cocoa butter and 2% added cholesterol for 5 months. Dietary calcium was varied at 0.08, 0.2, 1.2 and 2% of diet. Serum total lipids, phospholipids, cholesterol and triglycerides decreased significantly with increasing dietary calcium, often to levels equal to or below those of the control diet. Liver, heart and aortic lipids were unaffected. Fecal lipids increased significantly between the 0.2% and 1.2% calcium level, primarily because of an increase in the free fatty acids. Fecal bile acids rose significantly at the 0.2% calcium level, with no further increase on increased calcium. 3-β-Hydroxysterol excretion increased as calcium increased and was, at the higher calcium levels, found to equal all the exogenous cholesterol, whereas fecal lipid excretion was found to account for the weight gain differences between the various calcium levels. Histological examination of the kidneys indicated no pathological condition attributable to high dietary calcium.

Previous work in this laboratory has shown a calcium-mediated decrease in serum cholesterol and serum triglycerides in man (1) and rats (2). During these studies, fecal lipid excretion increased both in man [4] and in rats (2). At elevated dietary calcium levels, the serum cholesterol decrease in rats occurred concomitantly with an increase in fecal bile acid excretion (2). The literature on this has been reviewed by Yacowitz et al. (1).

The present study was carried out in an attempt to determine whether long-term feeding of calcium would produce the same hypolipemic effect of short-term feeding periods, as reported previously (2). A second purpose was to study the possible mechanism of the hypolipemic action of calcium.

EXPERIMENTAL

The basic experimental design, the methods of statistical analysis of the results and diet have been detailed previously (2). Thirty-two male 400-day-old albino rats of the Holtzman strain were divided into 5 groups. Four groups were fed the experimental diet with graded levels of calcium: 0.08%, 0.2%, 1.2% and 2.0%, respectively, as calcium carbonate, in a corn-soya ration containing 18% added cocoa butter and 2% added cholesterol, and 0.29% endogenous phosphorus. The fifth group of 8 rats were fed commercial, low fat rat pellets [5] and served as a normal rat control. The control diet contained 5% fat, 1.15% calcium and 0.76% phosphorus as determined by chemical analysis of the particular batch of rat pellets used. The rats were fed distilled water ad libitum. The experimental period was 5 months.

The analytical procedures for serum tissues and feces have been detailed (2), except that in this experiment cholesterol and triglycerides were analyzed on an auto-analyzer (3, 4) and fecal 3-β-hydroxy-

Received for publication September 6, 1966.

[1] This work was supported by Public Health Service Research Grant no. H-5905 from the National Heart Institute, by a grant from the Deering-Millikin Foundation, and by a grant from the Morris County Heart Association.

[2] Atherosclerosis Research Group.

[3] Health Research Institute.

[4] Yacowitz, H. 1962 Effect of dietary calcium upon lipid metabolism in man. Federation Proc., *21*: 258 (abstract).

[5] Control diet was Big Red Rat and Mouse Diet, Agway Inc., Syracuse, New York.

TABLE 1

Effects of dietary calcium and fat on serum and fecal lipids, mean weight gain and feces weight

	Calcium level in diet, %				
	Control diet	0.08	0.2	1.2	2.0
Serum lipids					
Total lipids, mg/100 ml	567 ± 8.3 [1]	1003 ± 18.0	772 ± 40.3**	644 ± 94.0**	450 ± 0**
Phospholipids, mg/100 ml	188 ± 10.6	255 ± 9.5	198 ± 6.5**	172 ± 12.2**	169 ± 5.8**
Cholesterol, mg/100 ml	163 ± 11.5	359 ± 12.6	287 ± 4.3**	240 ± 2.9**	168 ± 26.4**
Triglycerides, mg/100 ml	194 ± 8.1	272 ± 14.0	188 ± 13.1**	133 ± 4.5**	101 ± 3.6**
Mean wt, initial, g	497 ± 16.8	489 ± 14.7	506 ± 10.8	494 ± 15.5	504 ± 17.9
Mean wt, final, g	534 ± 19.5	631 ± 7.1	677 ± 27.3	550 ± 19.9	578 ± 24.3
Mean wt, gain, g	37 ± 18.4	142 ± 15.1	171 ± 19.2	56 ± 26.8	74.3 ± 36.4
Feces					
Dry wt, g/rat/day	7.0 ± 2.6	3.3 ± 0.6	4.4 ± 0.1	5.2 ± 1.1	5.4 ± 1.3
Wet wt, g/rat/day	15.5 ± 0.5	9.1 ± 1.5	10.1 ± 1.0	12.5 ± 0.5	11.1 ± 0.8
Ash, %, dry basis	19.4 ± 0.3	25.7 ± 1.7	27.8 ± 0.1	21.0 ± 0.7	18.6 ± 0.8
Sand, %, dry basis	3.3 ± 0.2	25.0 ± 2.7	22.1 ± 0.2*	7.5 ± 0.1*	0.6 ± 0.6
Calcium, %, dry basis	6.1 ± 1.1	1.1 ± 0.3	0.9 ± 0.02	4.5 ± 0.03**	8.4 ± 1.1**
Fecal lipids, mg/rat/day					
Total lipids	417 ± 62.2	448 ± 29.4	463 ± 0.8	969 ± 16.8**	1053 ± 54.5**
Lipid phosphorus [2]	5.5 ± 0.8	3.3 ± 0.2	3.9 ± 0.1	3.5 ± 0.2	5.1 ± 0.2**
3-β-Hydroxysterols [3]	20 ± 1.7	205 ± 23.2	222 ± 10.1	304 ± 34.1*	339 ± 39.9*
Triglycerides	57 ± 3.1	45 ± 4.4	52 ± 2.5	51 ± 12.5	53 ± 6.0
Bile acids	2.1 ± 0.2	6.7 ± 1.4	13.1 ± 0.4**	13.3 ± 0.2**	13.1 ± 0.4**
Free fatty acids, mEq/rat/day	0.45 ± 0.12	0.49 ± 0.07	0.67 ± 0.04	13.81 ± 0.30**	14.13 ± 0.28**

[1] Mean ± SD.
[2] Reported as phospholipids.
[3] Reported as cholesterol.
** Significance at $P = 0.01$.
* Significance at $P = 0.05$.

sterols were determined by the method of Vahoney et al. (5). Fecal neutral lipids, which were used in the determination of fecal free fatty acids, and fecal glycerides, were separated from pigmented contaminates by chromatography on silicic acid columns, using chloroform and diethyl ether as eluents. The total quantity of free fatty acids was then determined on the autoanalyzer (6), and the individual fatty acids within the free fatty acid fraction were determined by gas-liquid chromatography (2).

RESULTS

During the course of the experiment, one rat receiving the 0.08% calcium level, one rat, the 0.2%, and three rats, the 2% calcium level diets died. In all cases, autopsy indicated the cause of death to be respiratory infection. No specific reason was found for the number of deaths from respiratory infection in any group. Respiratory infections were common to all groups.

With the diets used, there was a greater weight gain at the 0.8% and the 0.2% calcium level than at the higher calcium levels (table 1). Rats fed the experimental diet gained equally well or better than those receiving the control diet. The mean feed consumption, corrected for feed wastage, was comparable in all groups fed the experimental diets. There was a trend toward increased fecal dry weight as calcium increased (table 1). In table 1 are shown the serum lipid values for the various diets. Serum total lipids, phospholipids, cholesterol and triglycerides significantly decreased, concomitant with an increase in dietary calcium. Although at lower calcium levels, increased dietary fat and cholesterol caused a serum lipid increase, at the 1.2% calcium level which is comparable to that of the control diet with respect to calcium, serum total lipids and phospholipids were not significantly different from the control level, whereas serum triglycerides were significantly lower than the control level ($P < 0.01$). Serum cholesterol remained significantly higher than the control at the 1.2% calcium level ($P < 0.01$).

There was no significant increase in fecal total lipids between the 0.8% and the 0.2% calcium levels (table 1), but at 1.2% calcium, a significant increase in fecal lipid excretion occurred ($P<0.01$). Increasing the calcium level to 2% did not cause a further significant increase in fecal lipids. Fecal bile acid excretion (table 1) showed a significant increase at the 0.2% calcium level ($P < 0.01$), with no further increase at higher calcium levels. This is a repetition of the results in the earlier short-term study (2). When the effect of dietary fat concentration at comparable calcium levels, i.e., control diet and 1.2% calcium levels, is examined (table 1) there is a sixfold increase in fecal bile acid excretion at the higher fat level ($P < 0.01$). The excretion of lipid phosphorus was unaffected by increasing dietary calcium, except at the 2% calcium level, or by increasing dietary fat levels (table 1). Excretion of 3-β-hydroxysterols showed an increasing trend with increase in dietary calcium ($P < 0.05$). This increase was concomitant with the decrease in serum cholesterol. A significant increase in the 3-β-hydroxysterol excretion was noted between the high fat diet at any calcium level and the control diet. This reflects the fact that the experimental diets contained 2% added cholesterol, whereas the control diet was devoid of exogenous cholesterol. Fecal glyceride excretion appeared to be independent of both dietary fat concentration and calcium concentration (table 1). Fecal free fatty acids (table 1) showed a significant increase at the 1.2% level ($P < 0.01$), concomitant with the large increase in fecal total lipid excretion, with no further significant increase at the 2% calcium level. As might be expected at comparable calcium levels, there was a significantly greater free fatty acid excretion with the high fat diet than with the control diet ($P < 0.01$). This, of course, is reflected in the large difference in total lipid excretion between the 2 diets.

Fecal calcium excretion did not show a significant difference at the 0.08% and 0.2% calcium levels, but at higher calcium levels, increased significantly ($P < 0.01$) as a function of increasing dietary calcium. The dietary fat concentration did not significantly influence calcium excretion. Sand excretion decreased

TABLE 2

Tissue lipids in rats fed graded levels of calcium

	Calcium level in diet, %				
	Control	0.08	0.2	1.2	2.0
			Liver, mg/entire tissue		
Total lipids	715 ±35 [1]	5806 ±848	5667 ±1004	4508 ±405	3346 ±1546
Phospholipids	242 ±11	3154 ±252	2942 ± 123	1827 ±175**	2091 ± 40*
Total cholesterol	77 ± 8.9	1873 ±191.7	2323 ± 394	1973 ±141.1	1043 ± 587.5
Triglycerides	61 ± 9.3	675 ± 80.6	835 ± 198.2	335 ± 45.8	538 ± 162.5
Free fatty acids, mEq/tissue	242 ± 3.5	977 ± 19.9	995 ± 9.4	944 ± 17.3	858 ± 133.5
Mean tissue mass, g	18.8 ± 1.2	27.5 ± 1.5	27.3 ± 1.6	21.6 ± 1.5	22.4 ± 2.8
Mean tissue mass, % of body wt	3.52	4.35	4.03	3.92	3.87
			Heart, mg/entire tissue		
Total lipids	44.8 ± 2.4	53.4 ± 3.9	54.9 ± 1.9	43.5 ± 2.3*	45.3 ± 2.2
Phospholipids	14.5 ± 0.7	17.7 ± 1.1	18.8 ± 1.0	13.9 ± 0.5*	13.4 ± 0.7*
Total cholesterol	3.8 ± 0.1	4.0 ± 0.2	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Triglycerides	2.7 ± 0.4	3.0 ± 0.9	3.7 ± 0.8	2.2 ± 0.6	2.8 ± 0.6
Free fatty acids, mEq/tissue	15.3 ± 0.5	15.9 ± 0.7	13.5 ± 0.9	14.0 ± 0.8	17.5 ± 0.6
Mean tissue mass, g	1.7 ± 0.03	1.9 ± 0.13	1.7 ± 0.06	1.7 ± 0.09	1.7 ± 0.04
Mean tissue mass, % of body wt	0.31	0.30	0.25	0.30	0.29
			Hair, mg/g		
Cholesterol	4.99 ± 0.14	7.21 ± 0.23	7.92 ± 0.19**	8.29 ± 0.09**	8.47 ± 0.15**
			Aorta, mg/g		
Cholesterol	3.09 ± 0.19	2.88 ± 0.15	3.14 ± 0.34	3.58 ± 0.41	3.08 ± 0.45

[1] Mean ± SD.

* Significant at $P = 0.05$.

** Significant at $P = 0.01$.

as a function of dietary calcium ($P<0.01$). This was to be expected, since sand replaced the calcium in the low calcium diets (2).

Tissue lipids are reported in table 2. No significant changes were noted in liver mass, total lipid, cholesterol, triglyceride or free fatty acids due to increase in dietary calcium. There was a decrease at the 1.2% calcium level in phospholipid ($P < 0.01$), with no further significant decrease at the 2% calcium level. All liver lipids were significantly higher with the high fat diet than with the control diet, regardless of calcium levels ($P < 0.01$). Similar results were noted with heart lipids, except that dietary fat concentration did not affect heart lipid concentration. Hair cholesterol increased with increasing dietary calcium ($P < 0.01$), but aorta cholesterol was unchanged (table 2).

Examination of serum free fatty acids (table 3) showed a progressive increase in the di- and polyunsaturated fatty acid ($P < 0.01$) with increasing levels of dietary calcium. There was some small decrease in the saturated fatty acids. Neither the serum triglyceride fatty acids nor the fecal fatty acids (for the latter, see table 3) showed any effect attributable to dietary calcium. Omentum fatty acids (table 3) also increased in P/S ratio with increasing dietary calcium. This is primarily due to increase in the monoenoic fatty acids with a decrease in saturated fatty acids. A deposition of monoenoic fatty acids from the serum free fatty acids into the omentum could account partly for the monoenoic fatty acid decrease in the serum free fatty acids.

DISCUSSION

The calcium levels used were selected to span a range from the minimal adequate level to a large excess. The commercial control diet contained approximately 1.2% calcium. The 0.08% calcium level represented the minimal obtainable calcium level as this was the endogenous calcium in the corn and soya used in the preparation of the diet. The 0.2%, 1.2% and 2% levels were approximately 2.5, 15 and 25 times the endogenous level. It had been reported previously (2) that although a large increase in fecal fat occurred at the 1.2% calcium level in a high fat diet, blood changes were noted at the 0.2% calcium level. The 2% level was included to learn whether a large excess of calcium caused stone formation or kidney damage. Both gross and histological examination of the kidneys indicated no pathological condition attributable to high calcium ingestion.

On a long-term basis, increased dietary calcium caused a decrease in serum lipids

TABLE 3

Effect of dietary calcium upon serum free fatty acids, fecal and omentum fatty acids

	Calcium level in diet, %			
	0.08	0.2	1.2	2.0
	mole % of total fatty acids			
Serum free fatty acids				
Saturated	30.85 ± 1.56 [1]	24.41 ± 3.05	22.26 ± 0.04	13.69
Monoenoic	30.83 ± 1.42	26.36 ± 4.85	16.03 ± 3.12**	9.75**
Di- and polyenoic	38.19 ± 2.22	50.91 ± 5.82	61.42 ± 2.90**	76.45**
P/S	1.26 ± 0.13	2.38 ± 0.48	2.76 ± 0.03**	5.58**
Fecal fatty acids				
Saturated	68.42 ± 1.16	70.15 ± 11.78	61.47 ± 6.25	72.67 ± 16.53
Monoenoic	24.53 ± 0.34	12.89 ± 6.24	16.15 ± 4.21	12.00 ± 6.44
Di- and polyenoic	6.98 ± 0.81	16.80 ± 5.49	22.23 ± 10.47	15.25 ± 0.07
P/S	0.10 ± 0.01	0.26 ± 0.12	0.38 ± 0.21	0.26 ± 0.20
Omentum fatty acids				
Saturated	33.30 ± 1.03	28.43 ± 2.88	16.78 ± 2.81**	17.07 ± 1.99**
Monoenoic	60.76 ± 1.03	66.01 ± 2.61	77.06 ± 5.09	72.51 ± 4.18
Di- and polyenoic	5.91 ± 0.68	5.70 ± 0.55	7.91 ± 1.49	10.39 ± 2.26
P/S	0.18 ± 0.02	0.21 ± 0.03	0.48 ± 0.03	0.60 ± 0.07**

[1] Mean ± SD.
** Significant at P = 0.01.

in rats fed a high level of a saturated fat and cholesterol. The serum lipid lowering was significant for all serum lipids examined. This extends the results of the previously reported short-term study (2), and indicates that the calcium-mediated lowering of serum lipids is not a transient effect but a persistent effect. Each increment of calcium caused a significant additional lowering of serum phospholipid, serum cholesterol, and serum triglycerides ($P < 0.01$), except that the cholesterol decrease between the 1.2% and 2% calcium levels was significant at $P < 0.05$, and the phospholipid decrease between 0.2% and 1.2%, and between the 1.2% and 2% calcium levels were significant at $P < 0.05$, respectively. Not only did calcium lower serum lipids with a high fat, high cholesterol diet, but it reduced the values to levels equal to or lower than the levels for the control rats which were not fed high fat or cholesterol.

An examination of liver, aorta and heart (table 2) indicated that no deposition of lipids occurred in the tissue. This confirmed the results of the short-term study (2) and indicated that the serum lipid-lowering effect of elevated levels of dietary calcium was not mediated by a transfer of lipids from the serum into the tissue. The decrease noted in tissue phospholipids at the two higher calcium levels may have been due to decreased phosphorus absorption in the presence of high calcium. This, however, requires further study.

The fatty acids in the serum free fatty acid fraction (table 3) became progressively more unsaturated as a function of increasing dietary calcium ($P < 0.01$). A similar effect was noted in omentum fatty acid ($P < 0.01$). In the serum free fatty acids, this appeared to be due to an increase in di- and polyenoic fatty acids at the expense of the monoenoic fatty acids, whereas in the omentum it appeared to be the result of a decrease in the saturated fatty acids ($P < 0.01$), concomitant with an increase in monoenoic fatty acids ($P < 0.01$). It appears that the increased unsaturation of the serum free fatty acids might be at least partially due to an exchange between the serum free fatty acids and the mobile pool of the depot fat as evidenced by the omental fatty acids (7).

Fecal fatty acids, with the diet used in this experiment, showed no variation in composition attributable to increased ingested calcium. In a previous report (2) in which beef tallow was used as the dietary fat, fecal fatty acids became progressively more saturated with increasing dietary fat. This difference in results may be attributable to the more highly saturated dietary fat, cocoa butter, used in this work.

In the earlier work (2) it was hypothesized that the serum cholesterol lowering was at least partially mediated through the excretion of bile acids in the feces. In the present study there was an increase in fecal bile acids at the 0.2% calcium level ($P < 0.01$), with no further increase at the higher calcium levels (table 1). Although fecal bile acid excretion could partially explain the cholesterol lowering at the lower calcium concentration, there were further significant decreases in serum cholesterol at the 1.2% and 2% calcium levels, with no additional increase in the excretion of fecal bile acids. The fecal 3-β-hydroxysterol shows a progressive increase as a functional of increased calcium ($P < 0.05$). Because two of the prime methods of elimination of cholesterol are fecal bile acids and fecal 3-β-hydroxysterol, it appears that the cholesterol was apparently eliminated as 3-β-hydroxysterol. It had been reported previously (2) that the average feed consumption of this particular strain of rats, at approximately 400 days of age and receiving this high fat diet, was 16.3 g/day. As the diet contained 2% exogenous cholesterol, the rats were ingesting approximately 326 mg of cholesterol daily. A comparison of the serum cholesterol of the rats fed the control diet, which contained no exogenous cholesterol, and the rats fed the 2% calcium level diet indicates no significant difference in serum cholesterol. At the 2% calcium level, the rats excreted 339 mg of 3-β-hydroxysterols and 13.1 mg of bile acids daily, or a total of 26.1 mg more than the cholesterol consumed (table 1). The control rats excreted a total of 20.0 mg of 3-β-hydroxysterol plus 2.1 mg of bile acids. It thus appears that a reason the serum cholesterol levels were similar in these 2 groups is that at the high calcium level, the exogenous cholesterol was excreted. Whether this

cholesterol was excreted without first being absorbed or whether it was absorbed and other cholesterol from the body pool was excreted is still to be determined.

A significant elevation is noted in the liver cholesterol between rats fed a high fat, cholesterol diet at all calcium levels and the control rats. Since no significant difference was noted in the liver cholesterol with the high fat diet which could be attributed to calcium, it appears that the lowering of serum cholesterol by calcium was not due to a deposition of the cholesterol in the tissue, and thus the hypocholesterolemic effect of increased calcium appears to be due to excretion of exogenous cholesterol. There are several possible explanations which might account for the increased liver cholesterol. The high concentration of very saturated dietary fat could possibly stimulate cholesterol biosynthesis in the liver. Also, the data do not permit a determination as to whether the exogenous cholesterol was excreted without being absorbed or whether it was first absorbed and an equivalent amount of cholesterol from the body cholesterol pools excreted. The answers to this can be determined only by isotopic experiments.

The excretion of fecal glycerides was independent of both dietary calcium level and dietary fat level (table 1). The large increase in fecal free fatty acids at the 1.2% calcium level ($P < 0.01$) occurred concomitantly with the large increase in fecal total lipids ($P < 0.01$). Between the 1.2% calcium level and the 2% calcium level, neither the increase in fecal total lipids nor that in fecal free fatty acids were significant. It appears that the increase in fecal total lipids could be ascribed to the increase in fecal free fatty acids.

Apparently the triglycerides were hydrolyzed to free fatty acids and eliminated as the calcium salt of the free fatty acids. The large increase in fecal free fatty acids between the 0.2% and 1.2% calcium levels could account for the serum triglyceride decrease between these 2 levels. However, there was a large significant decrease in serum triglycerides between the 0.08% and the 0.2% calcium levels, whereas the fecal free fatty acid excretion and the fecal total lipid excretion remained essentially unchanged (table 1). With the low calcium diets, the triglyceride not excreted was distributed as body fat, as seen from the large weight increase in the rats fed the low calcium diets. As the calcium level increased, the excess fat was excreted as free fatty acids, and the weight of the rats receiving the high fat diet approached the weight of the rats fed the control diet. The fecal lipid excreted at the 1.2% calcium level was 969 mg/rat/day, whereas at the 0.2% calcium level, it was 448 mg/rat/day. The difference was 521 mg/rat/day. Over a 150-day period, the rats fed the 0.2% calcium level retained 78.15 g more fat than the rats fed the 1.2% calcium level. The difference in final weight between the 2 groups was 81 g. This indicates that the ingested fat not excreted could be accounted for by the increase in weight with the low calcium diets.

From the data obtained, high levels of dietary calcium appear to have a persistent hypolipemic action in mature male rats and, from previously reported studies (1), a hypolipemic effect in man. The use of dietary calcium for the control of dietary-induced hyperlipidemia without adversely affecting weight appears to merit further investigation.

ACKNOWLEDGMENTS

The authors wish to thank Dr. S. G. Kahn for the determination of aorta cholesterols, and Mrs. Neal Elsasser and Dr. W. E. Smith for histological examination of the kidneys.

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A Histochemical Study of Embryonic Rat Liver in Avitaminosis E [1,2,3]

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ABSTRACT The histological and histochemical differences between the vitamin E-sufficient and -deficient rat livers during teratogenesis were investigated. To obtain abnormal embryos, after mating, female rats of the Holtzman strain that had been maintained with a vitamin E-deficient ration since weaning, were given 2 mg of *dl*-α-tocopheryl acetate by gavage on the tenth day of gestation. The same dose was given daily during the first 5 days of gestation to the control group to ensure normal embryogenesis. Normal and abnormal embryos were collected daily from day 15 of gestation onward and were prepared by appropriate methods to show histochemically the amount of acid and alkaline phosphatase, cholinesterase, lipase, lipid, lipofuscin, glycogen, mucopolysaccharide and iron. The results revealed that deficient livers often showed massive, central necrosis and an increase in the size of the Kupffer cells. Although cholinesterase, lipase, lipid and glycogen were reduced in the deficient livers, acid and alkaline phosphatase, iron and mucopolysaccharide were increased. The abnormal livers also showed deposition of lipofuscin, especially on the erythrocytes. It was concluded that prenatal vitamin E deficiency affected the normal, chemical development of the embryonic liver.

A relationship between dietary massive liver necrosis and tocopherol deficiency is known (1–6). Schwarz (7) attributed the lesions to oxidation-reduction activity of vitamin E and factor 3 which participate in the catalysis of electron transfer, such as the transfer of hydrogen from substrates of intermediary metabolism to the terminal acceptor oxygen. Piccardo and Schwarz (8), in their electron microscopic study of dietary liver degeneration, described disruption of nuclear membrane, changes in the endoplasmic reticulum and disappearance of mitochondrial cristae. Later, Schwarz (9) mentioned respiratory decline as indicative of a specific function of tocopherol in the maintenance of normal energy metabolism. Abell et al. (10) and Fite (11) described increase in stainable lipid and macrophages, nuclear pyknosis, widening of the sinusoids and regeneration in such livers. McClean (12) postulated that vitamin E-deficient rats could not reaccumulate potassium, and emphasized failure of ion transport as the basic lesion in dietary liver necrosis. The experiment of Zalkin and Tappel (13) showed tocopherol to be effective in inhibiting mitochondrial lipid peroxidation and hence its necessity as a lipid antioxidant. Dam (14) reported erythrocytic hemolysis and autoxidation of fatty acids in vivo in avitaminosis E.

None of these studies deals with embryonic liver. Preliminary histological observations on the liver of the term fetuses in avitaminosis E have been reported briefly.[4,5] The present work was intended to extend the previous histological study to the developing liver of the rat from day 15 of gestation onward and to report the histochemical changes in this deficiency as well. A total of more than 228 embryos was used for this purpose.

EXPERIMENTAL

Weanling female albino rats (Holtzman strain), weighing about 35 to 40

Received for publication August 1, 1966.

[1] Supported in part by Public Health Service Research Grant no. RG 4993 and no. HD 00634 from the National Institutes of Health and a Public Health Service General Research Grant to the University of Iowa College of Medicine.

[2] A preliminary report of the experiments appeared in Federation Proc., 25: 242, 1966 (abstract).

[3] Presented in part at the 7th International Congress of Nutrition, Hamburg, Germany, 1966.

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g, were fed the following vitamin E-deficient diet: 60% dextrose, 10% lard, 24% vitamin-free casein, 4% salt mixture (USP XIV), 2% cod liver oil and a vitamin mixture which provided in mg/kg diet: β-carotene, 3; pyridoxine, 5; riboflavin, 10; *p*-aminobenzoic acid, 10; thiamine·HCl, 15; niacin, 20; 2-methyl-1, 4-naphthoquinone, 25; Ca pantothenate, 50; pteroylglutamic acid, 50.5; inositol, 400; choline, 1000; vitamin D_2, 0.01; vitamin B_{12}, 0.05 and *d*-biotin, 0.30 (15). Food and water (distilled) were given ad libitum.

When the rats weighed about 175 g, they were mated with males maintained with a commercial stock ration.[6] To prevent total resorption of the embryos, a single dose (2 mg) of *dl*-α-tocopheryl acetate in corn oil[7] was administered by stomach tube to the experimental group on day 10 of gestation. The same dose of vitamin E was given daily for the first 5 days of gestation to the control group to ensure normal embryogenesis (15). In the experimental group nearly 22% of the embryos were saved from resorption by this treatment. Approximately 12% of these embryos exhibited various congenital abnormalities, such as intestinal hernia, exencephaly, ectocardia, and were used in this study. The remaining normal-appearing embryos were discarded. Sections 7 μ thick, from control and experimental embryos, after being fixed in respective fluids generally for 24 hours as required for the techniques, were cut and stained. Table 1 shows the methods employed and the ages of the embryos used.

RESULTS

Cholinesterase. Sites of cholinesterase activity in developing embryos have been reported.[8] The enzyme was discernible in the cytoplasm of the parenchymal cells. The nuclear membrane gave an intense reaction. Normoblasts, megakaryocytes, erythrocytes and endothelial cells were also positive. On day 20 of gestation Kupffer cells in the normal group gave a very intense reaction. The chromosomes seen in the mitotic figures were also enzyme-positive. No appreciable difference was discernible between the 2 groups on day 16. However, from day 17 onward, the quantity of the enzyme in the abnormal liver cells was conspicuously less than that in the normals (figs. 1 and 2).

Glycogen and PAS-positive substance. In neither group were PAS-positive granules observed in the liver up to day 17 of gestation, suggesting little or no glycogen formation up to this stage. From day 18 onward, glycogen was restricted to the cytoplasm of the hepatocytes in the normal liver. The PAS reaction in the abnormal liver was variable. Glycogen granules, as determined by β-amylase-treated slides, were fewer than normal (figs. 3 and 4). After β-amylase treatment more nonglycogenic PAS-positive substance was detected in the abnormal hepatic tissue, especially in the Kupffer cells (figs. 5 and 6). In a few older fetuses, agranular PAS-positive substance was seen in addition to the granular reaction for glycogen. The PAS-positive granules were not confined to the cytoplasm of the hepatocytes, but were seen in the intercellular regions also. Some of these granules were unusually large (figs. 3 and 4).

Iron. Blue-colored, iron-containing granules were seen in the Kupffer cells in both types. However, there was much more iron in the Kupffer cells of the abnormal livers, some of which also frequently contained phagocytized erythrocytes (figs. 7 and 8).

Acid phosphatase. Normoblasts, megakaryocytes, parenchymal, Kupffer and endothelial cells of the liver gave a positive reaction at day 16 in both groups. The quantity of the enzyme appeared to decrease with advancing gestation. There was more intense reaction in the older abnormal livers than in the normal ones (figs. 9 and 10).

Alkaline phosphatase. In the 13-day normal embryos, the liver became alkaline phosphatase-positive. The enzyme was present in the normoblasts, megakaryocytes, parenchymal and endothelial cells from day 16 onward in both groups. Intense reaction was observed in the regions surrounding the blood vessels. On

[6] Purina Laboratory Pellets, Ralston Purina Company, St. Louis.
[7] Mazola, Corn Products Company, Argo, Illinois.
[8] King, D. W., and M. Overturf 1963 Sites of cholinesterase activity in developing rat embryos. Anat. Rec., *145*: 330 (abstract).

TABLE 1

List of methods and ages of the embryos [1]

	Method	Final visual product	Gestational age
			days
1	Acid phosphatase: Gomori (34) Fixative: cold acetone Substrate: sodium glycerophosphate Paraffin section	PbS	16, 17, 20, 21
2	Alkaline phosphatase: Lillie (35) Fixative: cold acetone Substrate: sodium glycerophosphate Paraffin section	CoS	16, 17, 18, 20, 21
3	Cholinesterase: Gomori quoted by Barka and Anderson (36) Fixative: cold acetone Substrate: myristoylcholine Paraffin section	CoS	17, 19, 20
4	Lipase: Lillie (35) Fixative: cold acetone Substrate: Tween 40 [2] Paraffin section	PbS	16, 17, 18, 19, 20
5	Lipid: Gomori (34) Fixative formol-calcium Frozen section Stain: Sudan black B	lipid	15, 17, 19, 20, 21
6	Lipofuscin: Casselman (33) Fixative: 80% cold alcohol Paraffin sections Stain: Sudan black B	lipofuscin	21
7	PAS: McManus, cited by Pearse (37) Fixative: Rossman's fluid Paraffin section β-Amylase treatment for glycogen	glycogen and mucopolysaccharide	15, 17, 19, 21
8	Iron: Gomori, cited by Humason (38) Fixative: 10% buffered formalin Paraffin section Stain: potassium ferrocyanide	$Fe_4[Fe(CN)_6]_3$	21
9	Masson trichrome: Lillie (34) Fixative: Zenker Paraffin section	collagen, green; nuclei, red; rbc, yellow	15, 16, 20
10	Hematoxylin and eosin Fixative: Zenker Paraffin section	nuclei, blue; cytoplasm, pink	15, 16, 17, 18, 19, 20, 21

[1] For each technique, sections cut at 7 μ from at least three normal and three abnormal embryos were made.

[2] Polyoxyethylene sorbitan monopalmitate, Atlas Powder Company, Wilmington, Delaware.

day 17 of gestation, the enzyme was clearly demonstrated in the bile canaliculi (figs. 11 and 13). No appreciable difference was noticeable between the 16- to 18-day groups. The 20- to 21-day abnormal livers gave more intense reaction than the normal ones, however (figs. 11 and 12).

Lipofuscin. In the 21-day abnormal livers most of the erythrocytes in the central veins were heavily coated with this pigment, whereas those of the normal gave only a light reaction (figs. 14 and 15).

Lipid. The distribution of lipid in livers of 11- to 13-day embryos has been reported from this laboratory.[9] In this study very little lipid was observed in the

[9] Cheng, D. W., and L. F. Chang 1960 Distribution of lipid in the early vitamin E-sufficient, vitamin E-deficient and congenitally abnormal embryos and placentas. Anat. Rec., *136*: 312 (abstract).

TABLE 2
Summary of results

Substance	Observation
Cholinesterase	Less in abnormal liver
Lipid	Less in abnormal liver
Lipase	Less in abnormal liver
Glycogen	Less in abnormal liver
Nonglycogenic PAS-positive substance	More in the abnormal liver, especially in Kupffer cells
Lipofuscin	More in the abnormal liver
Iron	More in the abnormal liver
Acid phosphatase	More in the abnormal liver
Alkaline phosphatase	More in the abnormal liver

15-day embryos of both groups. The quantity, however, increased progressively with development. There was less lipid in the vitamin E-deficient liver of the abnormal embryos in comparison with the vitamin E-sufficient ones (figs. 16 and 17). In the 20-day vitamin E-deficient livers the lipid was seen concentrated mostly around the blood vessels in some erythrocytes, reticulo-endothelial, Kupffer and hepatic cells. The results of the histochemical work are summarized in table 2.

Lipase. In both groups, from day 15 onward, normoblasts, megakaryocytes, parenchymal and endothelial cells gave a positive reaction. The reaction in neutrophils was intense. The amount of enzyme appeared to increase progressively with gestation. In the abnormal liver it was consistently less than that in the normal ones (figs. 18 and 19).

In addition to the histochemical changes observed above, the abnormal livers often showed massive central necrosis (figs. 20 and 21). The parenchymal cells appeared less compact. Staining with hematoxylin and eosin and Masson trichrome revealed thinning of the nuclear membrane, breaking up of chromatin, reduction in the size of the nucleolus and increase of connective tissue in the abnormal liver. Furthermore, the sinusoids and Kupffer cells appeared larger in the vitamin E-deficient liver. Megakaryocytes, signaling the onset of thrombopoiesis, were recognizable as early as day 17 of gestation in livers of embryos of both types. In the 20-day abnormal livers blood platelets were also noted. Normoblast and bile ducts were conspicuous in both types, also.

DISCUSSION

The morphological changes that occur during mammalian development are better known than the biochemical events that affect such changes. Some of the biochemical changes and correlations between enzymic activity and developmental status have recently been reviewed by Herrmann and Tootle (16). Certain chemical determinations of the respiratory enzymes, such as cytochrome c reductases and cytochrome oxidase, in the liver of the normal prenatal rat have been carried out (17). The specific activities of all these enzymes increased two- to threefold from day 16 of gestation to term. Since vitamin E is shown to be a component of cytochrome c reductase (18), its deficiency will conceivably result in a diminution of this enzyme. Furthermore, since the electron-transport system plays a fundamental metabolic role in producing ATP required by many enzymic processes, a reduction of cytochrome c reductase will affect many enzymes.

Previous work from this laboratory (19) has shown that the average tocopherol level of the 21-day abnormal fetal liver is slightly, though not significantly, lower than normal (0.94 ± 0.94 vs. 2.53 ± 1.90 mg/100 g liver). The present study shows differences in enzymic reactions, in addition to histological changes, in the embryonic livers in vitamin E deficiency.

All primitive embryonic tissues and especially all nuclei at early stages contain phosphatases (20, 21). When the definitive form of a tissue is finally attained, the amount of the phosphatase

gradually diminishes. The higher-than-normal activity of the alkaline phosphatase in the vitamin E-deficient livers of older fetuses observed in the present study, may suggest a delayed maturation of this organ. Also, by the methods used in this study, nuclear diffusion artifacts could not be ruled out. Future studies using azo-dye coupling methods appear to be feasible.

Acid phosphatase is contained in lysosomes (22-26). The increase of such material in the older abnormal liver may reflect lysosomal accretion associated with necrosis.

Cholinesterase activity has been reported to decrease in vitamin E-deficient tissues (18). The reduced amount of this enzyme in the abnormal livers may result directly from vitamin E deficiency. However, erythrocytes are reported to have a high content of cholinesterase (27). Consequently, the anemia of the vitamin E-deficient abnormal embryos (our unpublished data) may also account for the diminution of this enzyme.

In vitamin E deficiency, plasma lipase is decreased (18). A lesser amount of lipase in the vitamin E-deficient livers may indicate less fat metabolism. Since the important feature of lipases is their readiness to catalyze the synthesis as well as the hydrolysis of fats and esters (28), the presence of less lipid in the abnormal group (fig. 17) may be correlated with the reduced lipase activity.

The reduced amount of liver glycogen in this deficiency is in accord with the observations of previous workers (3).[10] The increased quantity of nonglycogenic PAS-positive material in the liver may be correlated with the augmentation of connective tissue observed from sections stained by Masson trichrome connective tissue stain. Also, part of the nonglycogenic PAS-positive material in the parenchymal and Kupffer cells represent lysosomes (25). This further suggests an increased number of lysosomes. It is of interest to note that large PAS-positive granules seen in abnormal livers (fig. 4) resemble autophagosomes observed in connection with regeneration in the mammalian liver (29).

The increase of iron-containing granules in the Kupffer cells probably resulted from the hemolysis of the erythrocytes due to oxidation of their lipoprotein membrane. Recently Walker and Kummerow (30) observed erthrocytes of vitamin E-deficient rats to be susceptible to hemolysis by copper and iron, whereas Marvin (31) reported a reduction in the survival time of rat erythrocytes in avitaminosis E. Essner and Novikoff (32) think that iron-containing granules in human liver cells are derived from lysosomes.

The intensifying deposition of lipofuscin on the erythrocytes in the abnormal livers indicates the autoxidation of tissue lipid in the absence of vitamin E and is consistent with the observation of Casselman (33). According to him the necrotic tissues and blood vessels may play a role in the formation of lipofuscin (or ceroid pigment) that tends to coat the erythrocytes, which not only act as catalyst but also provide surface for the resulting product to deposit on. Further investigation is planned concerning the deposition of lipofuscin in avitaminosis E.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Professors N. S. Halmi and J. J. Kollros in reading the manuscript; the help rendered by A. N. Rao, M. Overturf, J. Scranton and R. Schults during the course of this work; and the technical assistance of E. Hirschler, L. Squire, D. S. Kelley and D. R. Brallier.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Section of a 20-day, vitamin E-sufficient liver demonstrating cholinesterase. Note the intense reaction in the Kupffer cells (arrow). × 700.

2 Section of a 20-day, vitamin E-deficient liver demonstrating cholinesterase. Note less enzyme in the Kupffer cell (arrow) than in figure 1. × 700.

3 Section of a 19-day, vitamin E-sufficient liver. Note the presence of PAS-positive glycogen granules in the cytoplasm of parenchymal cells and their absence from the nuclei (arrow). Periodic acid Schiff. × 700.

4 Section of a 19-day, vitamin E-deficient liver. Note reduced glycogen content. A few large PAS-positive granules (long arrow) are outside the parenchymal cells. Short arrow points to agranular PAS reaction. Periodic acid Schiff. × 700.

5 Section of a 19-day, vitamin E-sufficient liver after β-amylase treatment demonstrating nonglycogenic PAS-positive substance. Periodic acid Schiff. × 700.

6 Section of a 19-day, vitamin E-deficient liver after β-amylase treatment. Note the nonglycogenic PAS-positive substance in the Kupffer cells (arrow). Periodic acid Schiff. × 700.

7 Prussian blue reaction for iron in the Kupffer cells of a 21-day, vitamin E-sufficient liver (arrow). × 700.

8 Prussian blue reaction for iron in the 21-day, vitamin E-deficient liver. Note the increase of iron in the Kupffer cells (arrow). × 700.

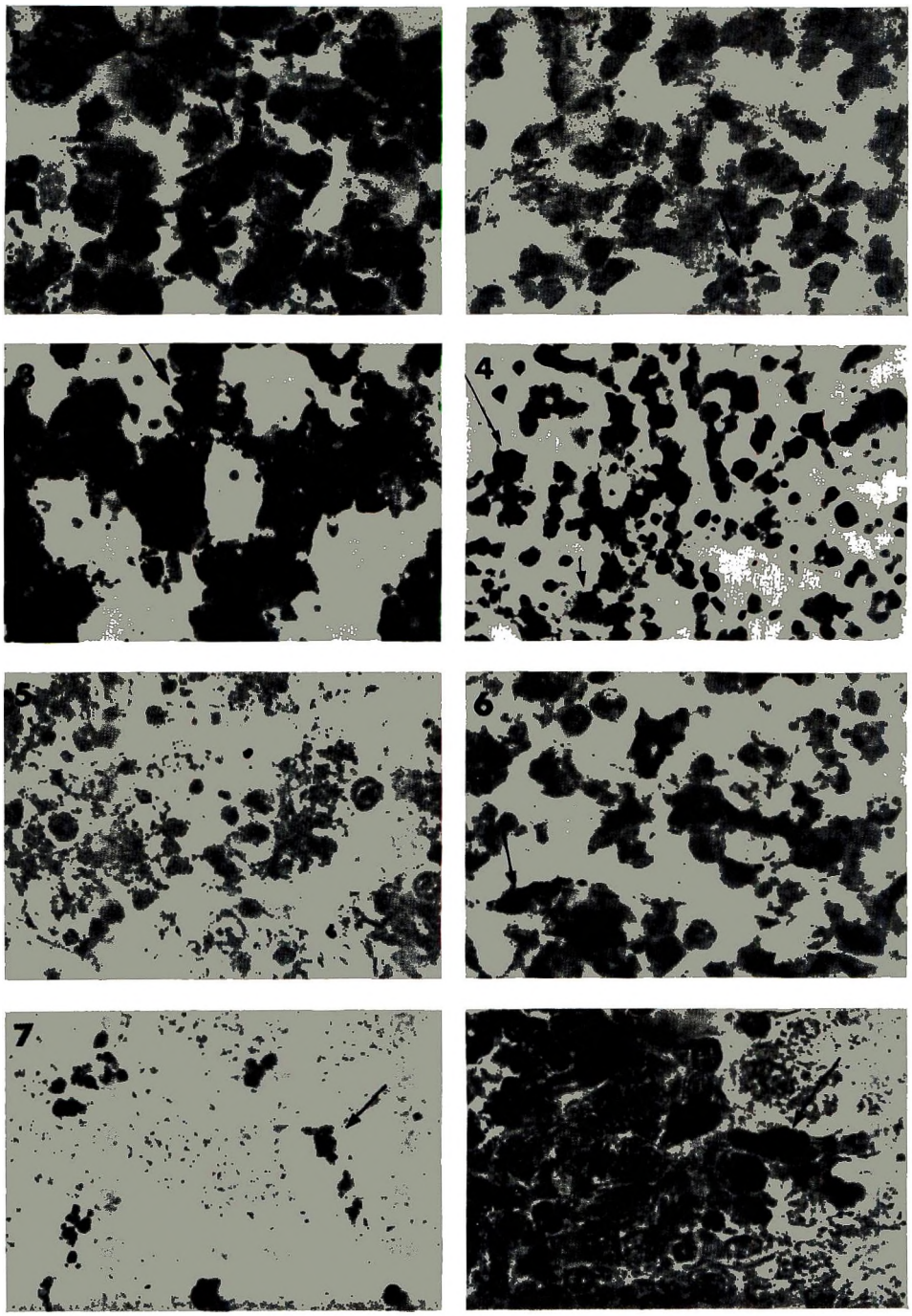
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PLATE 2

EXPLANATION OF FIGURES

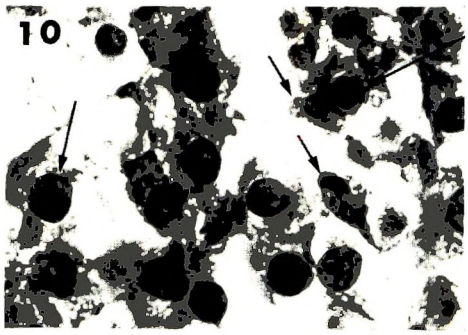
9 Section of a 21-day, vitamin E-sufficient liver demonstrating acid phosphatase. Note reaction in Kupffer cells (arrow). × 700.

10 Section of a 21-day, vitamin E-deficient liver demonstrating acid phosphatase. Note more enzyme reaction here than in figure 9 (small arrows, Kupffer cells; medium-size arrow, parenchymal cell; long arrow, normoblast). × 700.

11 Section of a 20-day, vitamin E-sufficient liver demonstrating alkaline phosphatase. Note the intense reaction around the blood vessel (arrow). × 400.

12 Section of a 20-day, vitamin E-deficient liver demonstrating alkaline phosphatase. Note more intense reaction here than in figure 11 (arrow). × 400.

13 Section of a 17-day, vitamin E-sufficient liver showing alkaline phosphatase reaction in the bile canaliculi (small arrow), and in the normoblasts (large arrow). × 400.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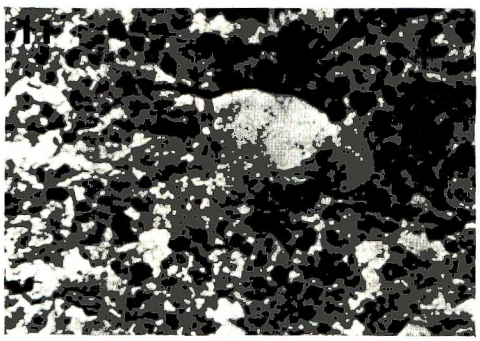

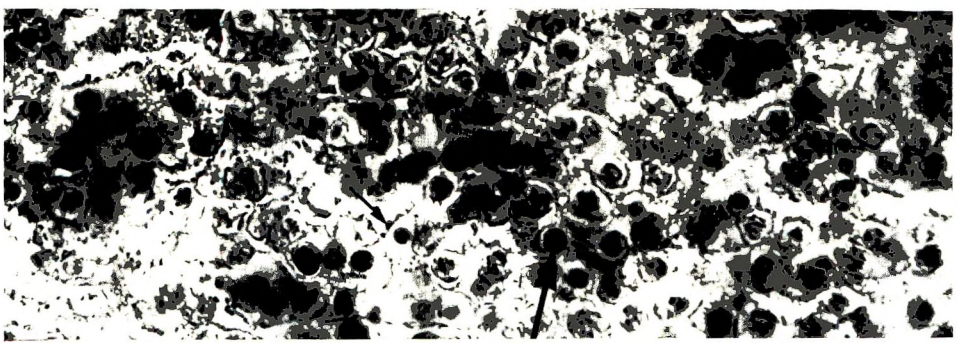

PLATE 3

EXPLANATION OF FIGURES

14 Sudan black B reaction in a 21-day, vitamin E-sufficient liver after xylene treatment of paraffin section demonstrating lipofuscin. Note the light coloration of erythrocytes in the central vein (arrow). Slight reaction is noticeable in the liver parenchyma. ×350.

15 Sudan black B reaction in a 21-day, vitamin E-deficient liver after xylene treatment of paraffin section. Note the intense black coating of lipofuscin on the erythrocytes in the central vein (arrow). × 350.

16 Section of a 21-day, vitamin E-sufficient liver demonstrating lipid (arrow). Frozen section, Sudan black B. × 700.

17 Section of a 21-day, vitamin E-deficient liver demonstrating lipid. Note less amount of lipid (arrow) here than in figure 16. Frozen section, Sudan black B. × 700.

18 Section of a 20-day, vitamin E-sufficient liver demonstrating lipase. The apparent black spots are neutrophils (arrow). × 700.

19 Section of a 20-day, vitamin E-deficient liver, demonstrating lipase. Note less enzyme here than in figure 18 (arrow points to a neutrophil). × 700.

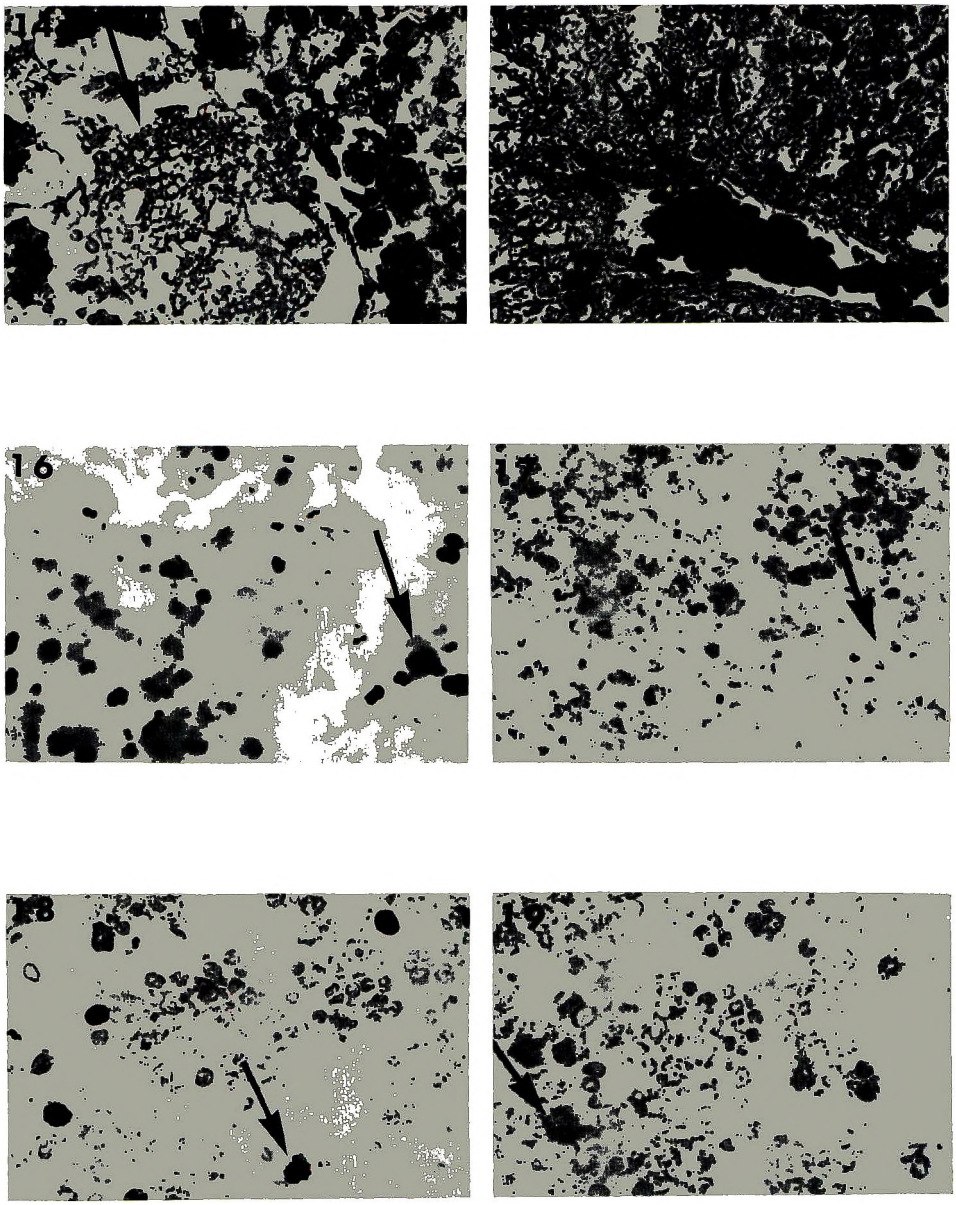
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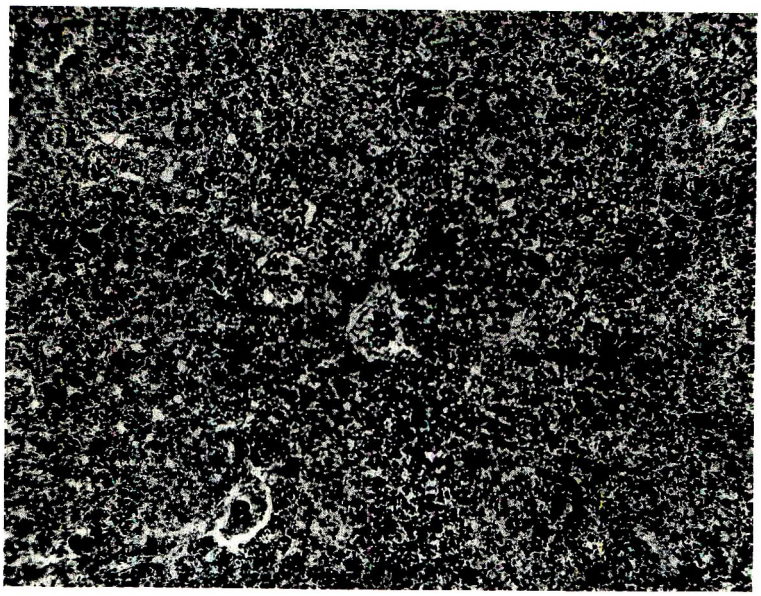
PLATE 4

EXPLANATION OF FIGURES

20 Section of a 21-day, vitamin E-sufficient liver showing normal appearance. H. & E. × 82.

21 Section of a 21-day, vitamin E-deficient liver showing central necrosis and widened sinusoids. A) Portal canal (arrow); B) central vein; C) liver parenchyma and D) hemorrhagic region. H. & E. ×82.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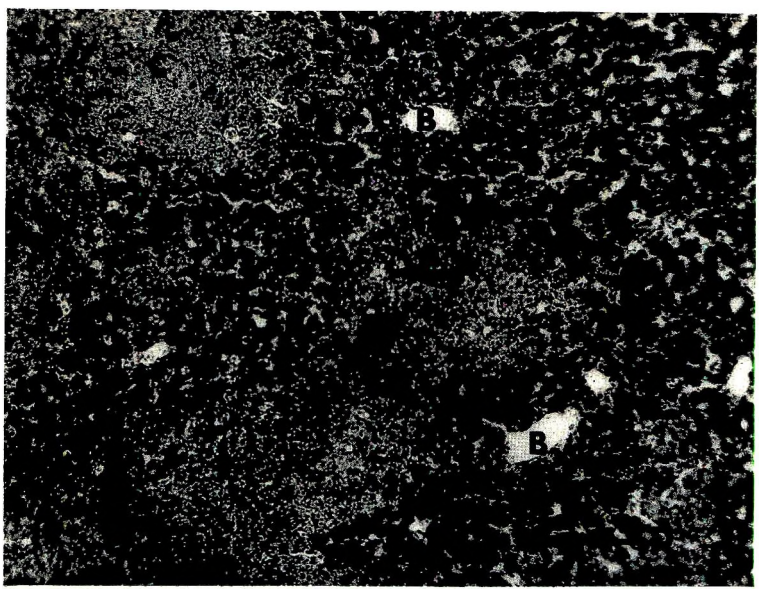
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B

Influence of Cyclopropenoid Fatty Acids on the Cholesterol Metabolism of Cockerels [1]

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ABSTRACT Cyclopropenoid fatty acids, as in *Sterculia foetida* oil and cottonseed oil, were fed to White Leghorn cockerels to determine whether these acids altered cholesterol metabolism. Corn oil was fed as a control. These cyclopropenoid fatty acids were found to cause increased plasma cholesterol, aortic atherosclerosis, liver weight, volume of bile in the gallbladder, bile acids in the bile, and bile acid excretion. The liver cholesterol concentration of the test cockerels was less than that of the controls. There was no difference in sterol retention, weight gain, or feed efficiency.

In 1957 Tennent and co-workers (1) compared the influence of several vegetable oils, including cottonseed oil, on the plasma cholesterol concentration and degree of aortic atherosclerosis of cholesterol-fed cockerels. In so doing, Tennent et al. used the equation of Keys et al. (2) to test whether an inverse relationship could be predicted between the polyunsaturated fatty acid content of the oils fed and the resultant plasma cholesterol concentration of the cockerels. This relationship held true with all the oils fed, except cottonseed oil. Cottonseed oil caused higher plasma cholesterol concentrations than predicted as well as a greater degree of atherosclerosis. Tennent and co-workers postulated that there must be a "substance of unknown identity" in the cottonseed oil which caused these unexplained elevations.

Among the oils tested by Tennent et al. (1), only cottonseed oil is known to contain the cyclopropenoid fatty acids. These acids, as well as other cyclopropene ring derivatives, are known to be the active agent(s) which causes marked changes in fatty acid metabolism and other deleterious physiological effects (3). We concluded that these acids could also be responsible for the unexplained effects noted by Tennent and his associates.

Reported here is a study of the effect of the cyclopropenoid fatty acids, as in cottonseed oil (approx. 1%) and *Sterculia foetida* oil (approx. 35%), on the cholesterol metabolism and aortic atherosclerosis of cockerels.

EXPERIMENTAL

Experiment 1. Day-old White Leghorn cockerels were fed a high protein (20%)-low fat (< 2%) initial diet for the first 8 weeks of life. This ration was of the same composition as the basal diet used in the experiment (table 1) except that grain sorghum was reduced to 59.6% and soybean meal increased to 30.0%. At the end of the seventh week, after culling, 80 birds were selected at random and separated into two 40-bird groups of comparable weight distribution. From 8 weeks of age until the end of the experiment each bird was given the basal diet and water

TABLE 1
Composition of basal diet

	%
Grain sorghum	64.55
Soybean meal (44% protein)	23.00
Dehydrated alfalfa meal (17% protein)	3.00
Vitamin and antibiotic premix [1]	2.50
Dried whey	2.00
Non-nutritive fiber [2]	2.00
Dicalcium phosphate [3]	2.00
Calcium carbonate	0.80
Sodium chloride	0.30
Methionine hydroxy analogue [4]	0.13
Manganese sulfate pentahydrate (70%)	0.02

[1] Supplied the following per kg of diet: (in milligrams) choline-HCl, 440.9; niacin, 27.6; chlortetracycline, 22.0; Ca D-pantothenate, 11.0; α-tocopheryl acetate, 5.51; riboflavin, 4.41; procain penicillin, 2.2; vitamin B_{12}, 0.013; and vitamin A palmitate, 9921 IU; and vitamin D_3, 1543 ICU.
[2] Solka-Floc, Brown Company, Boston.
[3] Dynafos, International Minerals and Chemical Company, Skokie, Illinois.
[4] MHA, Monsanto Company, St. Louis.

Received for publication July 22, 1966.
[1] Arizona Agricultural Experiment Station Technical Paper no. 1156.

TABLE 2

Effect of Sterculia foetida *oil on plasma cholesterol concentration (exp. 1)*

Dietary period	Dietary content of:		Oil fed by capsule (200 mg/day)	Plasma cholesterol	
	Corn oil	Cholesterol		2 weeks	4 weeks
	%	%		*mg/100 ml*	*mg/100 ml*
1	5	—	corn	114 ± 3.3 [1]	108 ± 2.7
	5	—	*S. foetida*	121 ± 2.5 *	122 ± 2.4 **
2	—	—	corn	99 ± 3.6	107 ± 4.8
	—	—	*S. foetida*	114 ± 2.6 **	135 ± 5.3 **
3	—	2	corn	288 ± 19.9	377 ± 28.0
	—	2	*S. foetida*	567 ± 42.8 **	686 ± 53.3 **
4	5	2	corn	529 ± 42.4	539 ± 40.8
	5	2	*S. foetida*	754 ± 66.0 **	685 ± 62.2 *

[1] Mean ± SE.
* Different from corresponding corn oil ($P < 0.05$).
** Different from corresponding corn oil ($P < 0.01$).

ad libitum and, daily, a gelatin capsule containing 200 mg of oil. The first group received 200 mg/day *S. foetida* oil and the second group received the same amount of corn oil each day as a control. Corn oil was obtained commercially and *S. foetida* oil was prepared as reported by Sheehan and Vavich (4).

The experiment was divided into four 5-week periods, during which the diets were rotated (table 2). Essential differences were variations in corn oil and cholesterol content of the diets. These diets were fed in an order such that inter-period carry-over was minimized. The diets used in each period were as follows: first period, basal diet in which 5% corn oil was substituted for 5% grain sorghum; second period, basal diet only; third period, basal diet in which 2% non-nutritive fiber was replaced by 2% USP cholesterol; and fourth period, 5% corn oil and 2% cholesterol were substituted as above. In addition, during the last week of the fourth period 0.2% chromic oxide was added as a marker to determine sterol retention. Feed consumption and chicken weights were recorded.

At the end of the second and fourth weeks of each dietary period the birds were fasted overnight and blood was taken by alar vein puncture and mixed with heparin. The plasma was separated by centrifugation and analyzed for total cholesterol by the method of Abell et al. (5). After the birds had been fed each of the four dietary variations, over 20 weeks in all, excrement samples were collected daily, for 3 days, from each bird and quickly frozen. Prior to analysis the excrement from each cockerel was thawed and mixed thoroughly. Excrement sterols, as cholesterol, were determined by the above method, chromic oxide by the method of Dansky and Hill (6), and total bile acids, as cholic acid according to Roscoe and Fahrenbach (7). At termination of the experiment each bird was weighed, killed by jugular exsanguination, and the liver, gallbladder, heart, and thoracic and abdominal aortas to the femoral branch were removed. The aorta was split longitudinally, stained according to Holman et al. (8), and graded by a committee of 4 associates by criteria set forth by Tennent et al. (9). Briefly, this system assigns numerical scores from 0 to 4, with the highest number signifying the greatest severity of atherosclerosis. Cholesterol content of the liver, bile, heart, and aorta was also determined by the method of Abel et al. Cholic and chenodeoxycholic acids were determined in the bile according to Mosbach et al. (10).

Experiment 2. The cockerels used in this experiment were raised and selected as in experiment 1. However, 80 birds were divided into 4 groups of 20 birds each. Again from 8 weeks of age until the end of the experiment each bird was fed daily a gelatin capsule containing 200 mg of oil.

The oil and cholesterol content of the ad libitum-fed diet was the same as that given during the fourth period of experi-

ment 1, i.e., 5% and 2%, respectively. The first group was given corn oil both in the diet and by capsule. The second group's diet was as the first except cottonseed oil was used instead of corn oil. The third group was given corn oil in the diet and a mixture of corn oil and *S. foetida* oil by capsule. Group four received corn oil in the diet and only *S. foetida* oil in the capsule. The quantity of *S. foetida* oil in the capsule of the third group varied from week to week in order to approximate the quantity of cyclopropenoid fatty acids consumed by the second group from cottonseed oil. The initial dose was 49 mg/day and was gradually increased to 110 mg/day near the end of the experiment. The quantity of corn oil was also varied in order to make the total oil content of the capsule up to 200 mg.

At 8 weeks, half of the birds, and at 16 weeks the remainder, were terminated and the aortas removed and examined as in experiment 1.

RESULTS AND DISCUSSION

Feed intake and body weight. Total feed intake and feed efficiency (feed consumed per unit weight gain) was similar for the groups throughout experiment 2 and all dietary periods of experiment 1. This shows that, for any comparisons made, the birds ingested similar amounts of oil or cholesterol; and that differences in cholesterol measurements between groups were not due to differences in cholesterol ingestion or caloric intake. Among all the groups of both experiments there were no significant body weight differences throughout.

Plasma cholesterol. In table 2 plasma cholesterol concentrations of the cockerels of experiment 1, at the second and fourth weeks of each dietary period, are presented. In all time periods and with all combinations of corn oil and cholesterol, the daily feeding of 200 mg *S. foetida* oil caused a significant ($P < 0.05$) to highly significant ($P < 0.01$) increase in plasma cholesterol concentrations.

Heart and aorta cholesterol. Visual grading or cholesterol analyses of the aortas from experiment 1 showed that the severity of aortic atherosclerosis (table 3) was greater ($P < 0.01$) after 20 weeks in the *S. foetida* oil-supplemented group than in the corn oil-supplemented control. Microscopic examination of cross sections of a few selected plaqued areas of the aortas of both groups verified the criteria used for visual identification of plaques. The greatest degree of heavy plaque formation, in both groups of cockerels, was observed in the area of the thoracic arch with less severe longitudinal plaques just above the femoral branch. There were no readily observable differences in the type of plaques between groups, just in degree of severity. The average cholesterol concen-

TABLE 3

Effect of the cyclopropenoid fatty acids in cottonseed oil and Sterculia foetida *oil on atherosclerosis of cockerels*

Amount and type of oil fed:				Aorta grades [1]			Aorta cholesterol conc
In diet		By capsule		8 weeks	16 weeks	20 weeks	20 weeks
%		*mg/day*					*mg/g*
				Experiment 1			
5,0,0,5 [2]	corn	200	corn			1.5 ± 0.17 [3 a]	4.4 ± 0.53 [a]
5,0,0,5	corn	200	*S. foetida*			2.3 ± 0.53 [b]	8.4 ± 0.43 [b]
				Experiment 2			
5	corn	200	corn	1.2 ± 0.29 [a]	1.5 ± 0.07 [a]		
5	cottonseed	200	cottonseed	1.0 ± 0.33 [a]	2.2 ± 0.43 [b]		
5	corn	200	corn + *S. foetida* [4]	1.6 ± 0.33 [a,b]	2.8 ± 0.51 [b,c]		
5	corn	200	*S. foetida*	2.2 ± 0.38 [b]	2.9 ± 0.45 [c]		

[1] Aorta grades or cholesterol concentrations are significantly different at a given time period if they do not have the same letter in the superscript.
[2] Represents percentage oil in the diet of 4 successive 5-week feeding periods.
[3] Mean ± SE.
[4] *S. foetida* oil provided cyclopropenoid fatty acids to this group equivalent to the average daily consumption of these acids by the group of birds consuming cottonseed oil.

TABLE 4

Effect of Sterculia foetida *oil on liver weight, sterol retention, and bile acid excretion (end of fourth period, exp. 1)*

	Capsule fed	
	Corn oil	*S. foetida* oil
Liver:		
Wt, g	29.8 ± 0.87 [1]	39.4 ± 1.07 **
Cholesterol, mg/g	28.5 ± 2.02	16.8 ± 0.85 **
mg/liver	861 ± 70	656 ± 41 **
Gallbladder:		
Intact organ [2] wt, g	6.2 ± 0.45	11.9 ± 0.08 **
Bile wt,[3] g	5.3	9.9
Bile composition: [3]		
Cholic acid:		
mg/ml	12.2	16.3
mg/gallbladder	62	155
Chenodeoxycholic acid:		
mg/ml	19.0	20.7
mg/gallbladder	97	197
Cholesterol:		
mg/ml	0.03	0.02
mg/gallbladder	0.17	0.19
Excrement:		
Bile acids, mg/g, dry wt	118 ± 30	234 ± 57 **
Sterol retention, as cholesterol, %	91.3 ± 0.51	90.6 ± 0.48

[1] Mean ± SE.
[2] Including bladder and bile within.
[3] Pooled average.
** Different from corn oil ($P < 0.01$).

tration of the entire heart tissue for both groups was 2.8 mg/g, wet weight.

In experiment 2 the feeding of 200 mg/day of *S. foetida* oil (table 3) again produced the highest degree of atherosclerosis, at both 8 weeks ($P < 0.05$) and 16 weeks ($P < 0.01$). The groups of cockerels fed corn oil, cottonseed oil, or the low level of *S. foetida* oil did not show significant differences from each other in severity of aortic atherosclerosis at eight weeks. However, at 16 weeks, significant differences among these 3 groups appeared. There was, in general, a direct relationship between the quantity of cyclopropenoid fatty acids fed and the severity of atherosclerosis found in the cockerels. Most noteworthy is that when the cockerels were fed cottonseed oil or the same quantity of cyclopropenoid fatty acids, as in *S. foetida* oil, for 16 weeks a greater severity of atherosclerosis was observed than when corn oil alone was fed. This is in line with the observations by Tennent et al. (1) for cottonseed oil. The unknown substance(s) in cottonseed oil mentioned by Tennent et al. are, in our opinion, the cyclopropenoid fatty acids.

Cholesterol content of liver and bile excretion. In experiment 1 the liver weight of the group fed *S. foetida* oil was higher ($P < 0.01$) than that of the corn oil-fed control. Both the cholesterol concentration in the liver and total quantity per liver was less ($P < 0.01$). This indicates that *S. foetida* oil caused increased depletion of cholesterol from the liver.

The gallbladders of the cockerels fed *S. foetida* oil were also enlarged ($P < 0.01$). This enlargement was due to a numerically increased volume of bile rather than larger tissue weight. The concentration of cholic and chenodeoxycholic acids of pooled bile samples was little different, whereas the total quantities were numerically higher in the gallbladder contents of the *S. foetida* group.

The higher concentration ($P < 0.01$) of the bile acids in the excrement of the treatment group fits into a hypothesis that

cyclopropenoid fatty acids cause increased depletion of cholesterol from the liver into the blood and bile (as bile acids). The sterol retention, as cholesterol, of both groups was not different.

ACKNOWLEDGMENTS

The authors wish to express appreciation to Dr. John M. Thomas for his advice and Ernest L. Nash, Jr. for his technical assistance.

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Cellular Response with Increased Feeding in Neonatal Rats [1]

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ABSTRACT Previous data have established that neonatal rats nursed in groups of 18 animals grew poorly and were ultimately smaller than normally nursed controls. This small stature was associated with fewer cells in all organs studied, both at weaning and at maturity. The size of individuals cells, however, remained normal. In the present study the cellular events associated with the known increased growth rate in rats nursed in groups of three to six animals per mother were examined. The results obtained demonstrated that the organs of these animals contain more cells than the organs of controls. Again individual cell size was normal. The increased growth rate and final size attained in these animals were a function of an increased rate of cell division. The combination of these data with the previous data suggests that the state of nutrition during the neonatal period is important to the rate of cell division and the final number of cells in the organs of the rat.

Rats nursed in large groups grow more slowly and attain a smaller final size than rats nursed in normal numbers (1). Previous data have demonstrated that this diminution in stature is associated with a reduced cell number in all organs studied (2). Cell size remains normal. Conversely, rats suckled in small groups grow more rapidly and attain a larger final size than controls nursed in normal groups (3–5). The cellular events associated with this increased growth rate and stature are unknown.

DNA content of diploid nuclei within a particular species is constant (6). Thus in any organ composed of diploid cells total organ DNA reflects cell number (7). Cell size may be expressed as a weight/DNA or protein/DNA ratio, and RNA per cell as an RNA/DNA ratio. Animals nursed in small groups were compared with littermate controls, using these criteria, in order to determine whether the increased size of various organs was associated with an increase in cell number, cell size, or both.

MATERIALS AND METHODS

Rats of the Sprague Dawley strain were used in all experiments. After weaning they were raised in separate cages and fed ad libidum amounts of a commercial laboratory chow.[2] Only male animals were used for tissue analysis. Nursing groups were arranged so that control mothers suckled six of their own offspring and six from an experimental mother. No differences in growth rates were noted in transferred pups. The experimental mother nursed either three or six of her own offspring. Thus littermates were nursed in groups of 12 animals and in groups of three or six animals. Comparison was made only between littermates.

Five animals from each group were killed at 21 days (weaning) and 130 days (adult). Organs were immediately dissected, weighed, and processed as described previously (2). DNA, RNA, and protein were extracted and determined quantitatively by techniques identical to those previously reported (8). Results are expressed as total quantities per organ.

RESULTS

There were no differences in any tissue analysis between organs of animals nursed in groups of three or six. Both of these groups have, therefore, been combined and considered the experimental animals. Each chemical determination was run in tripli-

Received for publication September 12, 1966.
[1] Supported in part by a grant from the Ross Laboratories, no. 15-0532082, the American Cancer Society IN-73D no. 2, and the Nutrition Foundation, no. 357.
[2] Rockland Rat Mouse Diet, Animal Feeds Inc., New York.

cate with less than 1% difference in matched samples. Each value represents the average of separate determinations in 5 animals. There was less than 10% variation of the method in whole animal and less than 6% in individual organs for animals within either experimental or control groups. There was no overlap of any values for either weight, protein, RNA or DNA when organs of experimental animals were compared with organs of control animals.

Weight. Table 1 demonstrates that by the time of weaning, the experimental animals were already significantly heavier than their littermate controls. This increase was reflected by increased weights of all organs. In the adult the whole animal and individual organs have maintained this increased weight. Although differences vary for individual organs, they are between 15 and 30%.

Protein. The increased weight in organs of experimental animals was paralleled by a proportional increase in total organ protein, both at the time of weaning and in the adult (table 2). Therefore, the increased wet weight described previously is associated with an actual increase in protoplasmic mass and does not merely represent edema.

RNA. RNA was similarly increased in all experimental organs both at weaning and in the adult. Again this increase is proportional to the increase in weight and protein (table 3). Thus there is a proportional increase in all cytoplasmic constituents.

DNA. DNA followed the identical pattern. Both at the time of weaning and in

TABLE 1

Weight of organs — control vs. experimental nursing groups of rats [1]

Tissue	At weaning		Adult	
	Control	Experimental	Control	Experimental
	g	*g*	*g*	*g*
Whole animal	59.10	64.20	376.40	402.80
Brain	1.49	2.01	1.88	2.21
Heart	0.36	0.44	1.42	1.74
Lung	0.39	0.44	2.21	2.67
Liver	3.14	3.87	12.03	14.81
Kidney	0.36	0.41	1.41	1.73
Thymus	0.33	0.45	0.72	0.94
Spleen	0.29	0.38	0.56	0.63
Submaxillary	0.17	0.22	0.60	0.71
Gastrocnemius	0.08	0.11	1.68	1.87

[1] Each value represents the average of separate determinations in 5 rats. There was less than 10% variation in whole animal and less than 6% in individual organs for animals within either experimental or control groups.

TABLE 2

Protein content of organs — control vs. experimental nursing groups of rats [1]

Tissue	At weaning		Adult	
	Control	Experimental	Control	Experimental
	mg	*mg*	*mg*	*mg*
Whole animal	5871	6324	—	—
Brain	96.1	147.0	306	344
Heart	58.1	69.3	272	310
Lung	41.3	48.7	557	597
Liver	437.5	492.0	4067	4826
Kidney	73.3	81.6	220	257
Thymus	45.3	57.1	127	160
Spleen	37.5	50.6	196	242
Submaxillary	17.5	21.9	60	81
Gastrocnemius	7.3	9.8	161	202

[1] Each value represents the average of separate determinations in 5 rats. There was less than 10% variation in whole animal and less than 6% in individual organs for animals within either experimental or control groups.

TABLE 3

RNA content of organs — control vs. experimental nursing groups of rats [1]

Tissue	At weaning		Adult	
	Control	Experimental	Control	Experimental
	mg	*mg*	*mg*	*mg*
Whole animal	109.30	132.60	—	—
Brain	3.46	4.25	4.16	4.97
Heart	0.92	1.28	2.14	2.62
Lung	2.21	2.64	4.53	5.34
Liver	19.26	23.15	95.72	119.61
Kidney	1.44	1.89	4.67	5.23
Thymus	1.07	1.66	1.44	1.97
Spleen	1.40	1.84	4.02	4.84
Submaxillary	2.54	3.03	4.47	5.06
Gastrocnemius	0.18	0.25	0.76	1.14

[1] Each value represents the average of separate determinations in 5 rats. There was less than 10% variation in whole animal and less than 6% in individual organs for animals within either experimental or control groups.

TABLE 4

DNA content of organs — control vs. experimental nursing groups of rats [1]

Tissue	At weaning		Adult	
	Control	Experimental	Control	Experimental
	mg	*mg*	*mg*	*mg*
Whole animal	97.81	126.20	—	—
Brain	2.18	2.54	3.10	3.72
Heart	0.62	0.912	1.43	1.98
Lung	1.96	2.28	3.34	4.05
Liver	4.96	6.01	25.68	30.60
Kidney	1.65	1.99	3.79	4.25
Thymus	2.74	3.14	1.47	1.81
Spleen	2.30	2.72	9.68	11.87
Submaxillary	1.17	1.39	1.94	2.59
Gastrocnemius	0.06	0.094	0.51	0.69

[1] Each value represents the average of separate determinations in 5 rats. There was less than 10% variation in whole animal and less than 6% in individual organs for animals within either experimental or control groups.

the adult there was an increase in whole animal and individual organ DNA content. With exception of brain this increase is again similar to that observed in weight, protein, and RNA. In brain the increase in DNA is proportionally less than the increase in weight, protein or RNA.

These data, therefore, indicate a proportional increase in all tissue elements studied. DNA is increased (table 4) indicating that all organs contain more cells. Weight, protein, and RNA are increased proportionally (except in brain). The ratios, therefore, remain unchanged, indicating that cell size is unaffected. Thus the animals nursed in small litters grow faster and attain a greater final size, which is reflected by an increase in cell number alone in most organs studied. The increased size of the brain is accompanied by a smaller increase in cell number with some increase in cell size as well.

DISCUSSION

Normal growth in the rat proceeds in a sequential manner. During prenatal and early postnatal development all organs grow by cell division alone. Gradually a shift occurs beginning in brain and lung and ending in skeletal muscle to increasing cell size as the major component of growth. For any individual organ 3 phases of growth can be described: hyperplasia, hyperplasia and concomitant hypertrophy, and hypertrophy alone (8). This general pattern is true in rat placenta (9) and in human placenta (10), the only human tissue studied.

Caloric restriction during the period of cellular hyperplasia results in decreased

growth, manifested by a decrease in cell number in all organs studied. Cell size is unaffected. The rate of cell division and the ultimate number of cells within a given organ may be reduced by limiting the number of calories available to the animal during the neonatal period (2).

The present study demonstrates that the reverse is also true: increasing the number of calories accelerated growth by increasing the rate of cell division. Again cell size remained unchanged. Ultimately these animals attained greater stature than controls, and their organs were composed of a larger number of cells.

Thus the rate of cell division and the ultimate number of cells that compose a particular organ are determined not only by intrinsic (genetic) factors but may be influenced by extrinsic (environmental) factors. The state of nutrition during the early neonatal period appears to be an important environmental factor.

These changes occur within certain limits. The data in this study demonstrate that at least as far as growth is concerned, a total of 6 animals nursing from a single mother appears to be optimal. Reducing litter size further does not result in an increase in weight gain or in the rate of cell division.

Data in the human infant suggest that undernutrition causes growth retardation (11) and that increased caloric intake may result in acceleration of weight gain in normal permatures (12). At present, the cellular events associated with these alterations in human growth patterns are unknown. It is possible, however, that similar effects on cell number will be found.

It is important to emphasize that these data are concerned with a description of cellular events occurring during accelerated growth. They in no way suggest either that the increased number of cells is physiologically beneficial to the animal or that the mechanism by which hyperplasia occurs is understood.

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Riboflavin Deficiency and Gastric Ulcer Production in the Rat: A procedure for the study of susceptibility to stress-induced gastric ulcers [1]

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ABSTRACT The influence of riboflavin deficiency on the induction of gastric ulcer in the rat was studied. A procedure was developed by which it was possible to study the susceptibility of rats of the Wistar strain to stress-induced ulcerogenic processes. The procedure was innocuous for the well-nourished rat. Riboflavin deficiency made the Wistar rat susceptible to induced gastric ulcer formation, and the administration of riboflavin protected the rat from these lesions. General inanition appeared to play a minor role in the susceptibility of rats to the ulcerogenic process when they were stressed by the procedure described.

The relationship between nutritional status and susceptibility to stress-induced gastric ulcer in the rat has not been studied. There have been some studies which have shown that diets inadequate with respect to some nutritionally essential component ultimately lead to some form of gastric abnormality. For example, Hoelzel and DaCosta (1) and Sharpless (2) have shown that a diet low in protein leads to hyperplasia with thickening and formation of ridges of the squamous epithelium of the forestomach.[3] The use of a diet consisting of white flour and salts by Howes and Vivier (3) led to a gradual reduction by 50% of food consumed by young rats with the production of grossly observable ulcers in the forestomach. When a limited quantity of the same diet was fed to adult rats, a majority of them also developed ulcers in the forestomach. A complete fast of 4 days' duration was observed by Robert and Nezamis (4) to lead to ulcer formation predominantly in the glandular portion of the stomach, but 50% of the rats developed ulcers in the forestomach as well (5).

The absence of gastric lesions in the rats studied by Wolbach and Howe (6) in their classic work on vitamin A deficiency in the rat appears to rule out this vitamin as one of importance in gastric ulcerogenicity. It can be assumed, however, that the diet used by these investigators was deficient in more than one vitamin, and a multiple deficiency may have altered the response of the gastric wall to the vitamin A deficiency. The absence of reports of a relationship between vitamin A deficiency and gastric ulcers during recent years, when the compositions of purified diets were more precisely known, lends support to the earlier observations. Howes and Vivier (3) reported that the animals fed the white flour diet did not develop hyperplasia of the forestomach if the diet was supplemented with 5% of its weight as whole yeast. Sharpless (7) observed that a mixture of riboflavin, nicotinic acid, cystine, and a rice polish concentrate was also protective. No beneficial effects were associated with the addition of thiamine. It was subsequently shown (8) that choline and pyridoxine, but not pantothenic acid, were also essential for the maintenance of a healthy squamous epithelium of the forestomach. Findlay (9) noted a papil-

Received for publication September 16, 1966.

[1] A preliminary report of this work was presented before the Meeting of the Federation of American Societies for Experimental Biology (Federation Proc., 25: 455, 1966), Atlantic City, New Jersey, 1966.

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[3] The forestomach has also been named the rumen, the prostomach, the proventriculus, the non-glandular portion, the cardiac portion, and the fundus. The glandular portion of the rat stomach has also been named the mucosal portion and the pyloric portion. The junction of the forestomach and the glandular portion is called the limiting ridge. The use of terms such as corpus, fundus, antrum, greater and lesser curvature introduces vagueness.

lomatous change in the forestomach of rats fed a diet low in riboflavin. This condition could be prevented by the administration of riboflavin but not by the addition of thiamine. A thiamine deficiency has been reported to lead to a high incidence of microscopic ulcerations in the forestomach and the glandular portion of the stomach, although no lesion could be found by gross examination (10). A deficiency of pantothenic acid appears to lead to duodenal ulcers in the rat (11).

When the question is raised as to whether epithelial hyperplasia, papillomatous change and microscopic lesions are gastric ulcers in the customary sense, it appears that any form of information relating nutrition to ulcerogenesis is scant. Apparently no studies have been made in the rat to relate nutritional status to stress-induced gastric ulcer susceptibility.

A number of techniques involving stressful situations are available for the induction of gastric ulcers in the rat. Those which are pertinent are, in a broad sense, physical stimuli in that the inducing stress as it is applied is a mechanical agent or procedure rather than a chemical one. These techniques are fasting (4), mechanical activity (12), subjection to cold (13), mixed changing position and intermittent photic stimulation (14), restraint (15), thermocautery (16), scalding (17), and pyloric ligation (18). Of these procedures, cold, mixed changing position and intermittent photic stimulation, restraint, thermocautery, scalding, and phyloric ligation cause the appearance of ulcers in the glandular portion of the stomach. The ulcers due to restraint do not penetrate the submucosa (19), and the thermocautery ulcers are intentionally placed in the glandular portion. Fasting may cause ulcers in the forestomach as well as the glandular portion (5), or only in the forestomach (4). Mechanical activity also causes ulcers in the intestine.

All of these procedures are capable of inducing ulcers in normal rats, and for this reason, none of them are well-suited for the purpose of differentiating between rats that have varying degrees of susceptibility to the induction of ulcers. It was necessary, therefore, to develop a method for stressing the rats which was incapable of inducing ulcers in normal rats, but which could induce their formation in susceptible rats. This is a report of the use of this procedure (Kim procedure) to demonstrate that rifioflavin deficiency renders the white rat susceptible to gastric ulcer formation.

MATERIALS AND METHODS

Weanling, female rats of the Wistar strain [4] were used. The conditions under which the animals were maintained have been described before (20). The diet consisted of the following: (in grams) vitamin-free sucrose, 680; vitamin-free casein, 180; corn oil, 100; and USP salt mixture no. 2, 40. To each kilogram of diet were added: (in grams) cod liver oil, 20; choline chloride, 1.5; and (in milligrams) pteroylglutamic acid, 0.6; biotin, 1.5; thiamine·HCl, 20; pyridoxine·HCl, 20; menadione, 50; nicotinamide, 50; potassium *p*-aminobenzoate, 50; Ca pantothenate, 60, and *i*-inositol, 100; and cyanocobalamin, 40 μg. When present in the diet, 20 mg of riboflavin were added per kg. Each rat received 5 mg of mixed tocopherols in corn oil once each week. The rats were assessed as deficient when they had failed to gain more than 5 g during a period of 14 days. All rats used were initially made riboflavin deficient; those to be used in the deficient state continued to receive the deficient diet, and those to be used in the normal state were fed the riboflavin-supplemented diet.

Several preliminary studies required for the design of the ulcer induction procedure will be summarized here. It was found that a mechanical device which rocked a cage at the rate of 160 cycles per minute gave optimal results. The cage consisted of 4 compartments, the sides and bottoms of which were solid and the top was hardware cloth. The compartment bottoms were covered with hardware cloth to provide a foothold. Two animals could be placed in each compartment, and in cases where paired animals were used, they were placed in the same compartment. The extent of a rocking excursion was 2.5 cm.

A considerable number of groups of rats was used; some groups were deficient and

[4] CFN rats, Carworth, Inc., New City, New York.

some had received riboflavin; some groups consisted of only 2 rats and some of 12 rats. A suitable stress was found to be a period of rocking of 2 hours' duration each day for 5 successive days. Examination of the stomachs of groups of riboflavin-deficient rats immediately after the sequential rocking exposures showed the following. After the second exposure, grossly observable dilatation of the capillaries and hyperemia of the glandular portion were noted; some edema of the forestomach and an occasional pinpoint ulcer could be seen in the glandular portion with the aid of a dissecting microscope. After the third exposure, the hyperemia and edema as described above were observed, but also a grossly observable minute ulcer in the glandular portion of 50% of the rats. After the fourth exposure, the same as the third, except that all rats had an ulcer, with some rats having multiple well-defined ulcers in the glandular portion. One rat had severe hyperemia but no grossly discoverable ulcer. After the fifth exposure, all rats had multiple grossly observable ulcers in the glandular portion. After the sixth exposure, the stomach contents of all animals were pigmented by blood as a result of hemorrhage from the multiple ulcers found. One animal had 10 ulcers, each of which was identifiable by a blood spot. After the seventh exposure, the observations were essentially the same as those following the sixth exposure, except that the average ulcer was larger; some were as long as 5 to 6 mm.

Histological sections through the ulcerated regions were examined by competent pathologists who confirmed the lesions as typical ulcerative processes. In none of the preliminary studies was an ulcer found in the forestomach; all ulcers were found in the glandular portion of the stomach. No ulcer was found in the duodenum.

The stomachs of a considerable number of riboflavin-deficient rats that were not subjected to a stress exposure were examined, and none of these stomachs were found to be abnormal. No examination was made of rats following a single stress exposure. It was not uncommon for a malnourished rat to die during the initial stress exposure, as will be seen in the following detailed report.

A stress of a 2-hour period of rocking on each of 5 successive days was used throughout the remainder of the studies, with the following exception. One large group of animals, who were permitted to eat a riboflavin-supplemented diet for 12 days after they had been classified as deficient, were stressed as described, but for 6 successive days. Examination of the stomachs of these animals after the sixth exposure showed, on gross examination, no abnormalities, but when examined under a dissecting microscope, a mild degree of dilatation of the capillaries in the glandular portion could be observed.

Following the establishment of a satisfactory procedure, 2 studies were undertaken. They were nearly identical in design, except that during the first (study 1) the animals were permitted to eat their respective diets ad libitum, whereas in the second (study 2), the paired-feeding procedure was used. Since study 2 is the more meaningful, it will be described.

Sixty female, weanling rats were fed the deficient diet until, progressively, some of them satisfied our criteria for riboflavin deficiency. As they became deficient, they were paired on the basis of their body weights and the pattern of the actual, although small, weight changes during the last 2 weeks. One rat of each pair was placed in the deficient group (group D) and the other member in the riboflavin group (group R). Twenty pairs of rats were used. The members of the 2 groups were paired-fed for 15 days; group D continued to eat the riboflavin-deficient diet ad libitum, whereas the members of group R were offered and consumed a quantity of the riboflavin-supplemented diet equal to the quantity of deficient diet eaten by the group D pair member. On days 11 through 15, the rats were stressed as described; both members of a pair were placed in a single compartment of the rocking cage. Following the fifth exposure, all rats were killed and the gastrointestinal tracts examined.

RESULTS

In study 1, all rats in group D continued to receive the riboflavin-deficient diet for 12 days following their having reached the deficient state. They were subjected to the

TABLE 1

Incidence of stress-induced gastric ulcers in riboflavin-deficient rats [1]

	Group D, riboflavin-deficient	Group R, riboflavin-supplemented
Avg wt of rats when assessed deficient, g	50 ± 3 [2]	50 ± 3
Avg wt of rats after 15 days of paired feeding, g	50 ± 3	53 ± 3
No. deaths during initial stress exposure (day 11)	3 [3]	2 [3]
No. rats with ulcers after stress exposure sequence (day 15)	14	2 [4]
No. rats without ulcers after stress exposure sequence (day 15)	1	13

[1] Twenty rats per group.
[2] An estimate of the standard error of the mean.
[3] Fifteen pairs remaining for evaluation.
[4] See text relative to inanition.

stress procedure during the next 5 days. None of these rats died during the initial stress exposure. All of the stomachs showed grossly observable ulcers in the glandular portion of the stomach. All the rats in group R, whose previous history was like that of the group D rats, were fed the riboflavin-supplemented diet ad libitum for 12 days, and they too were subjected to the stress procedure. The body weights of these young animals almost doubled during the 12-day period of riboflavin supplementation. None of these rats showed grossly observable abnormalities of the stomach, although the glandular portion appeared to be in a healthier state than that shown by the group D rats. The mucosa was also thicker than that seen in the group D rats.

In study 2, the members of pairs in the 2 groups were pair-fed for a total of 15 days. During the last 5 days of this period, they were subjected to the stress procedure. The stress procedure had no influence on the daily food consumption of the group D rats. The group D rats had an average body weight of 50 ± 3 [5] g at the beginning and at the end of the paired-feeding period. The group R rats attained an average body weight of 53 ± 3 g for a net gain of 3 g. During the initial stress exposure, 3 rats in group D and 2 rats in group R died. Examination of their stomachs showed hyperemia and capillary dilatation in the stomachs of the group D rats, but no ulcers in any case. The remaining 15 intact pairs of rats survived the stress procedure. Examination of the stomachs of the 2 groups of rats was made immediately following the fifth stress exposure.

Of the 15 rats in group D, fourteen showed extensive ulceration. One rat showed extensive capillary dilatation and hyperemia, but no grossly observable ulcer was seen. Of the fourteen showing ulcers, one had one large ulcer (8 mm) extending from the glandular portion through the limiting ridge and into the forestomach.

Of the 15 rats in group R, thirteen showed the usual non-ulcerous changes, but two of the rats showed very small lesions which we considered sufficiently abnormal to call ulcers. A summary of study 2 is presented in table 1.

DISCUSSION

The incidence of stomach and intestinal illnesses in Korea is a major medical problem and one which occupies a position of importance comparable to heart disease in the United States of America. Of these illnesses, the most frequently occurring is gastric ulcer. The fact that the condition occurs in young people, especially in the Korean Army, makes it a problem worthy of study. Our knowledge that the general level of nutrition in Korea is substandard, coupled with our conviction that the quantity of riboflavin is generally lower than suspected in such susbstandard diets, attracted our attention to this vitamin.

The fortunate combination of the selection of this vitamin for study, our experience in the study of the rat's requirement for riboflavin, and the finding of a stressing procedure which was apparently innocuous for the well-nourished rat make possible this report of the role of riboflavin deficiency in the susceptibility of the white rat to gastric ulcer formation.

The importance of the study is clear in terms of its being the first demonstration that the nutritional state of the animal in-

[5] Estimate of the SE of the mean.

fluences its susceptibility to an ulcerogenic process. The development of the Kim procedure for the induction of ulcers in susceptible animals appears to offer an opportunity to study a considerable number of factors for their role in this aspect of gastric physiology.

The deaths of the 3 rats in group D and two in group R of study 2 may have been due to the poor nutritional state of these animals, the former being deficient in riboflavin, the latter being deficient in caloric intake. Death may also have been caused by the failure of these rats to make the initial adjustment required for adaptation. The death of animals under an initial exposure to stress is commonplace, but such stresses are far more severe than the one used here. The absence of such deaths in study 1 may have been due in part to the shorter period of actual riboflavin deficiency (group D) or the excellent state of nutrition (group R).

The development of small lesions in 2 rats of group R in study 2 undoubtedly supports the concept that some susceptibility to ulcerogenic processes may stem from general inanition. The 2 rats in group R that showed these lesions were the two smallest of the group (40 g). They were almost starved, because their diet consumption was established by their pair-mates in group D, whose weights of 40 and 42 g place them as two of the four smallest in group D. These 2 group D rats showed 5 large ulcers with blood-stained gastric contents and 6 ulcers with hemorrhagic spots, respectively. It appears, however, that general inanition, while significant, plays a minor role.

The ulcers which were produced under these circumstances have been described by pathologists as typical ulcers. Although they appear to be strikingly similar to small, unperforated gastric ulcers in human beings, the 2 lesions may be etiologically unrelated. An interest in the incidence of gastric ulcer in man prompted this study. The results do not prove that riboflavin deficiency is a causative factor in ulcer formation in man, nor that ulcers can be cured by the ingestion of additional quantities of the vitamin, riboflavin.

ACKNOWLEDGMENTS

Sincere thanks are given to Drs. R. B. Wilson and P. K. Greening of our Pathology Department for evaluation of histological sections.

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Histidine Metabolism in the Human Adult: Histidine blood tolerance, and the effect of continued free L-histidine ingestion on the concentration of imidazole compounds in blood and urine [1]

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ABSTRACT Relationships among the intermediary metabolites of histidine were studied in the human adult. Histidine and imidazolelactic acid were the only imidazole compounds identified in the fasting plasma of 3 healthy human adults fed a constant diet. Plasma histidine tolerance studies in which 5 g of L-histidine (free base) were fed, showed the peak increase in histidine to occur one hour after ingestion; imidazolelactic acid changes paralleled that of histidine. Eleven imidazole compounds were quantitatively identified in 24-hour urine samples collected for periods of 3 consecutive days before histidine ingestion, during ingestion of a 10-g daily load of free L-histidine, and after histidine ingestion. Histidine loading caused an increase in the excretion of histidine, urocanic acid, imidazolepropionic acid, imidazoleacetic acid and imidazolelactic acid; none of these returned to the pre-histidine ingestion level by the end of the 3-day post-histidine period. The amounts of 4-amino-5-imidazole carboxamide, carnosine, homocarnosine, anserine, 1-methylhistidine, and 3-methylhistidine either were not affected by histidine loading, or the results were erratic and varied among the subjects.

Metabolism of histidine may proceed through a variety of pathways, although in mammalians the major amount of the amino acid is believed to be degraded via urocanate to glutamate (1). One of the more comprehensive studies in vivo of the urinary intermediary metabolites of L-histidine in adult man was made by Brown et al. (2). The investigation, although yielding valuable data, was primarily a comparative study of the patterns of the various metabolites in the urine of 3 different species: man, monkey, and the rat. Differences among the species in the pattern of radioactive-labeled intermediates were evident.

The present investigation with healthy human adults was designed to study 1) the concentration of imidazole compounds in blood and urine while a constant diet was fed; 2) the blood tolerance curve after a free L-histidine load; and 3) the changes in concentration and pattern of imidazole compounds in blood and urine after continued ingestion of a test load of free L-histidine added to the constant diet. Data are presented concerning the relationships among the intermediary metabolites of L-histidine in the human adult.

EXPERIMENTAL

Subjects. Three healthy adults, 2 female (G and E) and one male (A), with normal liver and kidney function served as subjects. They ranged in age from 22 to 30 years; their weights, which remained constant throughout the study, were 60, 74, and 87 kg, respectively. They lived in the Clinical Research Unit of the Medical Center during the study.

Diets. The constant diet fed contained 35 g of protein daily; 32.5 g were derived from milk, and the rest came from a special low protein, yeast-leavened bread (3), and lettuce. Other foods such as a commercial beverage,[2] canned fruit, butter, and jelly, were included to ensure an adequate caloric intake for the subjects. The diet was planned so that calories from fat

Received for publication September 2, 1966.
[1] Supported in part by Public Health Service Research Grant no. AM-06825-03 and no. 5 M01-FR-42-06 from the National Institutes of Health.
[2] Tang, a sweetened, ascorbic acid-containing concentrate, General Foods Corporation, White Plains, New York.

TABLE 1
Histidine tolerance of healthy human adults [1]

Subject	Hours after histidine ingestion				
	0	1	2	4	5
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
G	0.96 [2]	12.04	7.26		1.76
A	1.30	9.75	7.38	5.13	
E	0.77	11.58	6.96	3.53	

[1] Five grams of L-histidine (free base) dissolved in 100 ml of water containing 16 g of a commercial beverage (Tang, General Foods Corporation) constituted the test dose.
[2] Concentration of the amino acid in plasma.

and from carbohydrate made up 42 and 52%, respectively, of the total calories in the diet. The mineral mixture of Leverton et al. (4),[3] 3.6 g, was given daily in a portion of the diluted commercial beverage; a multivitamin supplement [4] also was fed daily.

Plan of study. The subjects were allowed an adjustment period ranging from 4 to 10 days while eating the constant diet. Adjustment was judged by nitrogen balance and maintenance of body weight. The 3 days following the adjustment period, the subjects were fed only the constant diet. This constituted the pre-histidine, or first control, period. During the fourth through the sixth day, 10 g of L-histidine, free base, (dissolved in the diluted commercial beverage) were fed daily, in 3 equal portions, in addition to the constant diet. During the seventh through the ninth day, the subjects again received only the constant diet; this constituted the post-histidine, or second control, period.

Histidine tolerance test and collection of blood samples. On the fourth day, breakfast was withheld, and blood was withdrawn from the cubital vein of the subject. Free L-histidine, 5 g, dissolved in 100 ml of water containing 16 g of the commercial beverage, was given orally. Blood was withdrawn at 1-, 2-, and 4-hour (5-hour for subject G) intervals. The food omitted at breakfast was incorporated into the noon and evening meals along with 2.5 g of free L-histidine at each meal.

Fasting samples of blood also were withdrawn on the fifth and sixth days of the investigation.

The blood was centrifuged, and the plasma prepared for analysis of amino acids and diazotizable compounds by the method of Stein and Moore (5); the extracts were stored at −20° until analyzed.

Collection of urine and feces. Throughout the study, 24-hour urine collections, kept under refrigeration, were made; aliquots were stored at −20° until analyzed. Samples were prepared for analysis of histidine, 1- and 3-methylhistidine and anserine by the method of Stein (6); and for the analysis of diazotizable compounds, the method of Block et al. (7) was used.

Fecal collections were pooled in periods of 4 days, using carmine markers, and frozen until analyzed.

Collection of foods. A replicate of the daily diet for each subject was slurried in a blender and frozen until analyzed.

Methods of analysis. Histidine, 1- and 3-methylhistidine, and anserine in the plasma extracts and in urine were analyzed by the method of Spackman et al. (8); diazotizable imidazoles, other than histidine, were determined by the method of Block et al. (7). Nitrogen determinations were made on aliquots of urine, feces, and food by the method of Scales and Harrison (9).

RESULTS

Plasma. The results of the histidine blood tolerance studies are shown in table 1. One hour after ingestion of 5 g of L-histidine, free base, the amount of histidine in the plasma increased approximately 7.5, 12.5 and 15 times that of the fasting amount for subjects A, G, and E, respectively. At the 2-hour interval, the amount of plasma histidine for all subjects was less than for the one-hour sample. The amount continued to decrease over the period of study, but did not return to the fasting level by the end of the tolerance test (table 1).

[3] Prepared by Nutritional Biochemicals Corporation, Cleveland.
[4] Decavitamins, Roerig Division, Chas. Pfizer and Company, New York.

Ingestion of free L-histidine for 3 consecutive days caused an increase in the amount of the amino acid in the fasting plasma on days 2 and 3 for all the subjects. No subject was able to decrease the amount of the fasting level of histidine on days 2 and 3 to the pre-histidine fasting level (day 1), although there was approximately a 12-hour overnight period between withdrawals of blocd when no histidine was given (table 2). After the initial increase on day 2, continued daily ingestion of L-histidine caused no substantial increase in the amount of the amino acid in the fasting plasma on day 3 (table 2).

Imidazolelactic acid was present in the fasting plasma, but in amounts too low to quantitate. During the tolerance test, the amount changed in direct relationship to the amount of histidine present. The amount in the fasting plasma remained essentially the same during the 3 consecutive days of histidine ingestion.

After histidine loading, the plasma contained an unidentified, diazotizable substance that was eluted from the chromatographic column approximately 80 minutes before the appearance of imidazolelactic acid. The amounts of this substance paralleled the content of plasma histidine during the tolerance test. It was not present in the fasting plasma during the 3 consecutive days of histidine ingestion.

Urine. The amounts of 11 imidazole compounds in the urine of the 3 subjects during the pre-histidine, histidine, and post-histidine periods are shown in table 3. Ingestion of 10 g of free L-histidine for 3 consecutive days caused an increase in the excretion of histidine, urocanic acid, imid-

TABLE 2

Concentration of free histidine in fasting plasma from healthy human adults ingesting 10 g of the amino acid for 3 consecutive days [1]

Subject	Days		
	1	2	3
	mg/100 ml	*mg/100 ml*	*mg/100 ml*
G	0.96	1.32	1.36
A	1.30	3.07	3.27
E	0.77	1.34	1.40

[1] See footnote 1, table 1. An additional 5 g of histidine, divided evenly between the noon and evening meals were given after the end of the tolerance test on day 1; on days 2 and 3, the amount was divided evenly between the 3 meals. The histidine routinely was dissolved in a diluted commercial beverage (Tang, General Foods Corporation).

TABLE 3

Effect of free L-histidine ingestion on the excretion of imidazole compounds by healthy human adults [1]

Imidazole compound	Subject G			Subject A			Subject E		
	Pre-histidine period [1]	Histidine period [1]	Post-histidine period [1]	Pre-histidine period	Histidine period	Post-histidine period	Pre-histidine period	Histidine period	Post-histidine period
	mg/24 hr	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*
Histidine	35.5	116.8	45.4	68.5	308.0	108.3	53.3	372.3	61.3
Urocanic acid	2.7	3.7	3.2	2.7	6.1	4.9	2.2	3.8	2.0
Imidazolepropionic acid	0.2	3.0	1.3	> 0.0	0.4	> 0.0	0.2	1.0	0.4
Imidazoleacetic acid	1.1	3.1	1.9	> 1.2	8.2	> 3.2	0.8	6.6	2.0
Imidazolelactic acid	5.1	12.0	8.9	5.0	12.5	11.4	5.0	17.5	13.8
4-Amino-5-imidazole carboxamide	9.2	10.2	5.5	4.5	8.0	5.3	> 0.0	nc [3]	> 0.0
Carnosine	1.8	2.2	1.2	3.4	5.2	3.0	> 2.1	2.7	> 2.1
Homocarnosine	1.7	1.7	1.2	> 0.0	> 0.0	> 0.0	> 0.0	> 0.0	1.1
Anserine	2.2	9.4	2.4	2.4	> 0.0	> 0.0	3.3	4.3	3.6
1-Methylhistidine	2.6	2.3	1.9	4.8	4.5	4.6	3.5	3.7	3.3
3-Methylhistidine	23.6	25.6	23.4	35.2	39.0	39.4	19.2	18.9	18.1

[1] During the pre- and post-histidine periods the control diet only was given. See footnote, table 2, for the histidine period.
[2] Average of 3, 24-hour consecutive urine collections.
[3] Not calculated for technical reasons.

azolepropionic acid, imidazoleacetic acid and imidazolelactic acid. In all but 2 instances (urocanic acid, subject E; imidazolepropionic acid, subject A) the amounts of these compounds remained elevated during the post-histidine period, and did not return to the pre-histidine period levels.

Histidine excretion was increased 3-, 4.5- and 7-fold by subjects G, A, and E, respectively, during histidine ingestion. The increase in urocanic acid excretion ranged from onefold (subject G) to twofold (subject A); and imidazolepropionic acid excretion from fourfold (subject A) to 15-fold (subject G). Imidazoleacetic acid excretion increased over a range of threefold (subject G) to eightfold (subject E); and imidazolelactic acid excretion from 2.5-fold (subject G) to 3.5-fold (subject E).

The excretion of the other 6 imidazole compounds either was not affected by histidine ingestion, or the results were erratic and varied from subject to subject (table 3).

Traces of unidentified, diazotizable compounds in the urine were eluted from the chromatographic column at 100 and 115 minutes after the elution of imidazoleacetic acid in both the pre- and post-histidine periods. The amount of the substance eluted at 115 minutes increased sharply during the 3 consecutive days of histidine ingestion. The excretion of the second unknown compound was not affected by histidine ingestion.

Nitrogen balance. All the subjects were in positive N balance or in equilibrium (N output within ± 0.5 g of N intake) during the pre-histidine period. During the period of histidine ingestion, subjects G and E retained 1.3 g and 1.2 g, respectively, of N daily, and subject A essentially was in equilibrium with a loss of 0.1 g daily. During the post-histidine period, subject G was in negative N balance for the first 2 days, and in equilibrium on the third day; subject A was in negative N balance, and subject E in N equilibrium throughout the entire period.

DISCUSSION

The metabolism of histidine in the mammalian may involve oxidative or transamination pathways with the formation of such intermediates as imidazolepyruvic acid, imidazoleacetic acid, and imidazolelactic acid; degradation via the urocanic acid pathway; or incorporation into dipeptides (e.g., anserine, carnosine) (1). There also is some evidence that direct methylation of histidine can occur in one species, the rat (10).

In the present investigation, analysis of the plasma after the subjects were loaded with histidine revealed histidine and imidazolelactic acid as the only identified imidazole compounds present. Even at the peak concentration of histidine in the plasma (one hour after ingestion of the amino acid), no other imidazole compounds were identified, although the combination of analytical methods used (7, 8) allows the identification and quantitation of 12 compounds containing the imidazole nucleus. Thus from the blood studies alone, it might be assumed that the main metabolic pathway of histidine in the intact, healthy human adult is one of deamination to the lactic acid derivative of histidine. Urine from the same subjects, however, contains increased amounts of urocanic acid and imidazolepropionic acid after histidine ingestion. These latter compounds indicate that degradation to urocanate and related compounds is another metabolic pathway. The relative importance of the 2 pathways is difficult to assess in the intact human adult. Neither the rate of utilization of the intermediates of the pathways, nor the rate of renal clearance (with the exception of histidine and 1-methylhistidine (11), and of urocanic acid (12) are known.

Under the conditions of the present investigation, ingestion of a histidine load did not cause increased excretion of the histidine-containing dipeptides, the methylated histidine compounds, or the carboxamide derivative of histidine.

In the histidine tolerance study, the peak concentration of the amino acid was reached one hour after histidine ingestion. This agrees with studies by others who fed 5 g or less of the amino acid to control subjects (13, 14).

The reason for the sustained increase in histidine concentration of fasting plasma during the 3 consecutive days of histi-

dine ingestion is not known. The overnight fasting period of 12 hours appears to be adequate to clear the blood to the pre-histidine fasting levels since the renal clearance for histidine is substantially greater than that for other amino acids with the possible exception of taurine, glycine, and 1-methylhistidine (11).

Brown et al. (2) have reported the presence of histidine, 1-methylhistidine, and imidazoleacetic acid in urine collected for 5 hours after the oral administration of 10 μCi of uniformly labeled L-histidine-^{14}C, dissolved in orange juice, to human subjects. No urocanic acid-^{14}C was detected. Other than histidine, 1-ribosylimidazoleacetic acid contained the greatest amount of radioactivity. Of the ingested radioactive material, 2.9% was recovered in the urine. Only 4 of the imidazole compounds analyzed for in the present investigation were determined by Brown et al., and comparison between the amounts found in the 2 studies is impossible because of the short time of urine collection in their investigation, and the difference in the units used to express the amounts.

Previous work from this laboratory (15) indicated that excretion of carnosine was increased somewhat by the ingestion of free-L-histidine. In the present study no consistent changes in carnosine excretion were noted when the subjects were loaded with 10 g of free L-histidine for 3 consecutive days. The difference in results between the 2 studies is believed to be one of methodology. Carnosine was determined by reaction with ninhydrin in the first study (8), whereas in the present work, the diazotization reaction of Pauly (7) was used. Unpublished data indicate the latter method to be more consistently accurate in the recovery of a wide range of known amounts of carnosine added to urine.

The presence in normal human urine of urocanic acid (16–18), imidazolepropionic acid (19), imidazolelactic acid (20), and 4-amino-5-imidazole carboxamide (21, 22) have been reported by various laboratories. All but the latter compound were found to be influenced by histidine loading.

The observations of the above workers are confirmed in the present study in which the simultaneous analysis of a number of imidazole compounds were determined quantitatively in both urine and blood collected from each subject who lived under metabolically controlled conditions and served as his own control. Before the advent of the automated procedure for the analysis of imidazole compounds (7), such a study was not feasible.

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Growth of Young Rats after Differential Manipulation of Maternal Diet

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ABSTRACT By manipulations in the diet of pregnant and lactating rats we have attempted to define a critical component which, when deficient, results in the previously reported growth-stunting of the progeny. Our previous experiments involved 50% overall restriction of dietary intake. We have now observed the effect of restoring, in sequence, certain dietary components to control levels. Restoration of vitamins and minerals failed to influence the effect of restriction. Restoration of the caloric intake by the addition of sucrose resulted in less marked but still significant growth-stunting. The conclusion that protein is the critical dietary component is given further support from results when mothers were fed, ad libitum, diets containing zein or Alpha Protein; even though the caloric intake of the mothers was equal to or greater than that of the control mothers receiving a good protein, the progeny were consistently stunted.

Dietary restriction of rats during mating, gestation and lactation is known to cause permanent growth-stunting in the progeny despite the latter receiving an unlimited food supply after weaning (1, 2). Subnormal efficiency of food utilization (the ratio of weight gained to food consumed), abnormally high nitrogen excretion and marked aminoaciduria are also observed consistently (3). All these effects are more pronounced in male progeny. The observations were made on rats of the McCollum strain in this laboratory and have been confirmed in the Sprague-Dawley strain. In previous studies the above effects were obtained by overall restriction of the maternal diet, a standard commercial preparation.[1] The studies reported in the present paper were designed to determine whether any particular dietary component is crucial in producing the growth-stunting effect.

PROCEDURE

Animals. Male and female rats from the McCollum colony of this laboratory were raised to the age of 5 to 6 months with a commercial stock diet,[2] fed ad libitum. Sets of 3 females, each weighing 240 to 260 g, were mated from 10 PM to 9 AM with one male, weighing 340 to 380 g. Mating was continued each night until sperm were seen on examination of vaginal lavage. The females were then housed individually. For each experiment assignations to the treatment groups were made in rotation according to the order of conception. The nutritional status of the animal was equivalent at the start of the experiments.

Experimental feeding. In this series of studies a laboratory-formulated diet was used exclusively, whereas previous work in maternal nutrition at this laboratory has centered around quantitative manipulation of the commercial diet. The formulated diet consisted of 20% casein, 72% sucrose, 4% fat, 4% minerals,[3] 2 ml of a fat-soluble vitamin solution[4]/kg of diet and 10 ml of a water-soluble vitamin solution[5]/kg of diet. Unless stated otherwise the experimental feeding was begun im-

Received for publication August 17, 1966.

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis. Composition, as provided by the manufacturer: (in %) crude protein not less than 23.0; crude fat not less than 4.5; crude fiber not more than 6.0; ash not more than 9.0. Ingredients: meat and bone meal, dried skim milk, wheat germ meal, fish meal, animal liver meal, dried beef pulp, ground extruded corn, oat middlings, soybean meal, dehydrated alfalfa meal, cane molasses, animal fat preserved with BHA, vitamin B_{12} supplement, calcium pantothenate, choline chloride, folic acid, riboflavin supplement, dried brewer's yeast, thiamine, niacin, vitamin A supplement, 0.5% defluorinated phosphate, 0.5% iodized salt, 0.07% ferric ammonium sulphate, 0.2% manganese sulphate, and a trace of zinc oxide.

[2] See footnote 1.

[3] Hegsted et al. J. Biol. Chem., *138:* 459, 1941 (Hegsted salt mixture, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

[4] α-Tocopherol, 5 g; percomorph oil (vitamin A 3,000,000 USP units, vitamin D, 425,000 USP units), 50 ml; vitamin D, 456 mg; and corn oil to make 435 ml.

[5] Consisted of: (in grams) Ca pantothenate, 2; niacin, 5; inositol, 10; *p*-aminobenzoic acid, 25; choline chloride, 100; biotin, 10; and (in milligrams) thiamine, 200; riboflavin, 300; pyridoxine, 250; folic acid, 20; and cyanocobalamin, 5; dissolved in 50% alcohol to a final volume of one liter.

TABLE 1

Effect of maternal restriction of a formulated diet on growth of offspring

Maternal diet	No. of litters	No. of pups born alive	Mean body wt on day after birth	No. of each sex at age 3 weeks	Mean body wt		
					At age 3 weeks	At age 27 weeks	At age 48 weeks
			g		*g*	*g*	*g*
Formulated diet (ad libitum)	3	28	6.6 ± 0.01 [1]	11 males	45.9 ± 1.6	443.7 ± 5.7(8) [2]	511.9 ± 5.6(8)
				14 females	46.4 ± 0.85	239.8 ± 4.9(14)	—
Formulated diet (50% restriction)	3	33	5.2 ± 0.06	9 males	22.6 ± 0.8	359.0 ± 8.3(8)	420.5 ± 10.3(8)
				13 females	22.6 ± 0.7	226.3 ± 5.4(13)	—

[1] Mean ± SE of mean.
[2] Numbers in parentheses indicate number of animals surviving to given age.

TABLE 2

Effect of supplementing a restricted maternal diet with vitamins and minerals on growth of offspring

Maternal diet	No. of litters	No. of pups born alive	Mean body wt on day after birth	No. of each sex at age 3 weeks	Mean body wt	
					At age 3 weeks	At age 14 weeks
			g		*g*	*g*
Formulated diet (ad libitum)	5	46	6.0 ± 0.07	17 males	35.8 ± 2.4	349.3 ± 10.2(17) [2]
				17 females	35.3 ± 2.1	226.5 ± 5.4(17)
Formulated diet with double quantity of minerals + vitamins (50% restriction)	6 [3]	49 [3]	5.5 ± 0.08 [3]	14 males [3]	18.8 ± 1.3 [3]	286.7 ± 10.6 [3](14)
				20 females	17.4 ± 0.6	184.8 ± 4.3

[1] Mean ± SE of mean.
[2] Numbers in parentheses indicate number of animals surviving to given age.
[3] These figures include one litter containing 2 males only. Pups nursed in litters of 3 or less are known to grow conspicuously faster than pups nursed in litters of 8, even when the mother is restricted. Both these males attained near-normal body weight.

mediately after mating, continued throughout gestation, and terminated on day 21 of lactation. At this time all mothers were again fed the commercial diet ad libitum. In the experiments involving dietary restriction, the ad libitum intake of the control group of animals was measured daily; 50% of the intake of the controls was fed to the restricted group on the following day.

Raising of the progeny. Litter size and body weight were recorded within 15 hours of delivery. Body weights were then measured each week. Weaning always took place in the fourth week after delivery; the exact time depended upon the size of the most retarded pups. All groups in a given experiment were weaned simultaneously. After weaning, all progeny were caged individually and offered ad libitum amounts of the commercial diet. In some experiments, long-term study was confined to male progeny because of their greater vulnerability to maternal dietary restriction, shown in previous experiments (1).

Overall restriction of formulated diet. A preliminary experiment was carried out to confirm that maternal restriction of the formulated diet produced growth-stunting in the progeny similar to that observed with maternal restriction of the less-well-defined commercial diet used in the earlier studies by this laboratory. Control animals were fed ad libitum, whereas 50% restriction was used with the experimental mothers. Data relating to the outcome of pregnancy are shown in table 1. The growth of both the male and female progeny of the restricted mothers was retarded; the degree of retardation is comparable to that previously observed following restriction of the commercial diet. The formulated diet was therefore regarded as suitable for use in defining the critical dietary components.

Vitamins and minerals. The next experiment was designed to test whether the diminished intake of vitamins and minerals which necessarily accompanies overall restriction is important in producing a restriction effect in the progeny. The formulated diet was again fed ad libitum to the control mothers; the experimental mothers were restricted by 50% but in this case the formulated diet was mixed with double the quantity of vitamins and minerals. The body weights at various ages (table 2) were consistently and considerably lower in the progeny of restricted mothers than in control progeny, in spite of an equal intake of vitamins and minerals. It is inferred that the regularly observed general restriction effect is not primarily attributable to the concomitant restriction of minerals and vitamins.

Calories. The hypothesis that the restriction effect is due to caloric deficit in the mother was tested by the use of 3 diets. Control animals were fed the commercial diet ad libitum. One experimental group was fed the commercial diet restricted to 50% of the control intake. The second experimental group was also fed the commercial diet at a 50% level of restriction but sucrose was added to render the total intake approximately isocaloric with the control group. Measurements of the actual caloric intake in early and late pregnancy and in gestation are shown as averages for each group in table 3; at each stage the intake, relative to body weight, was nearly equal in the control and sucrose-supplemented restricted animals. The results shown in table 4 indicate that restoration of the caloric intake to or beyond the level

TABLE 3

Mean caloric intake of pregnant and lactating rats on three dietary regimens

Diet	Gestation		Lactation
	Days 1–8	Days 9–21	Days 1–21
	kcal/100 g body wt/day	*kcal/100 g body wt/day*	*kcal/100 g body wt/day*
Commercial diet (ad libitum) [1]	29	34	65
Commercial diet (50% restricted)	15	21	35
Commercial diet (50% restricted) + sucrose	29	32	67

[1] See footnote 1 of text.

TABLE 4

Effect of supplementing a restricted maternal diet with sucrose on growth of offspring

Maternal diet	No. of litters	No. of pups born alive	Mean body wt on day after birth	No. of each sex at age 3 weeks	Mean body wt		
					At age 3 weeks	At age 12 weeks	At age 20 weeks
			g		*g*	*g*	*g*
Commercial diet [1] (ad libitum)	3	30	6.2 ± 0.06 [2]	10 males 6 females	41.2 ± 1.3 35.0 ± 2.0	338.8 ± 5.2(9) [3] 229.8 ± 5.9(6)	443.1 ± 8.7(9) —
Commercial diet (50% restricted)	4	41	5.8 ± 0.07	16 males 14 females	17.2 ± 0.4 16.2 ± 0.6	260.6 ± 4.2(12) 170.5 ± 4.1(8)	357.9 ± 6.5(12) —
Commercial diet (50% restricted) + sucrose	4	35	6.4 ± 0.18	17 males 13 females	31.6 ± 0.9 28.0 ± 0.8	308.4 ± 8.1(14) 196.7 ± 4.4(13)	392.4 ± 9.5(14) —

[1] See footnote 1 of text.
[2] Mean ± SE of mean.
[3] Numbers in parentheses indicate number of animals surviving to given age.

of the control animals did not prevent the occurrence of a restriction effect, although the data suggest that the effect was to some extent mitigated by the addition of sucrose. When the experiment was repeated at a later date the growth of the respective progeny (fig. 1) confirmed the earlier results.

At this stage of the study, it was felt that sufficient evidence existed to form the hypothesis that protein is the critical dietary component with respect to the restriction effect. This hypothesis is consistent with some beneficial effect from sucrose supplementation of restricted animals since the additional calories would be expected to reduce diversion of protein to use as a source of energy (4).

Protein. If protein is in fact the critical component, a restriction effect should oc-

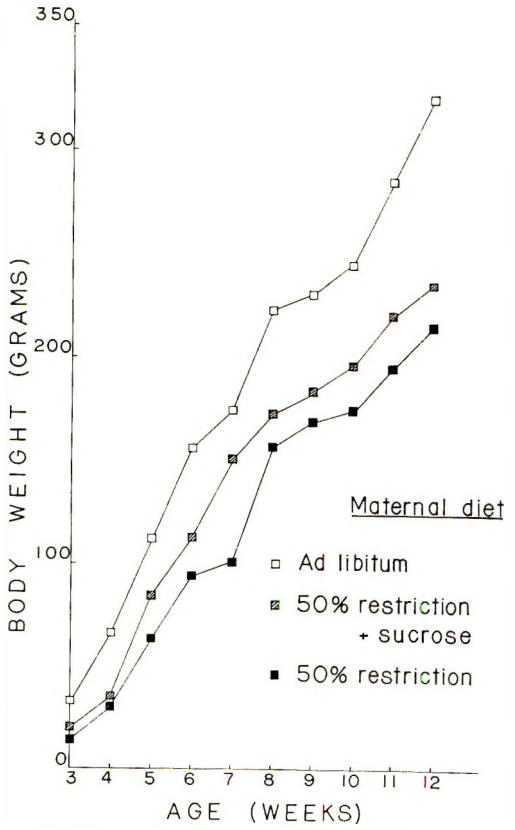

Fig. 1 Effect of caloric content of the maternal diet on the growth of the progeny. Open squares, mean body weights of 3 litters. Hatched squares, mean body weights of 5 litters. Solid squares, mean body weights of 4 litters.

cur when the mothers are fed a protein of low quality, even in ad libitum quantities. As a first test of this hypothesis, 3 diets were used. Control animals received the formulated diet. One experimental group received the formulated diet with zein substituted for half of the casein. The other experimental group received the formulated diet with zein substituted for all of the casein. This overall protein content was 20% in each case and all diets were fed ad libitum. In this instance the duration of experimental feeding included the mating period. Estimation of the food in-

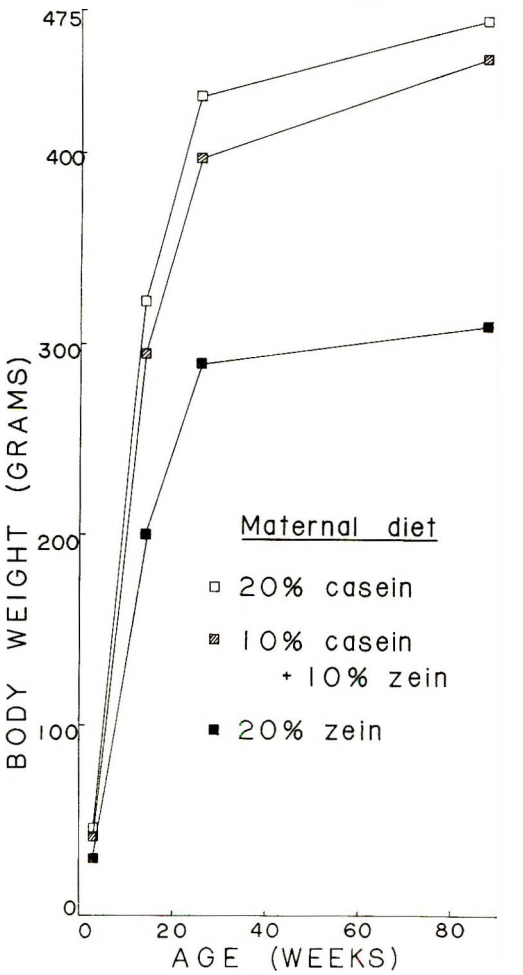

Fig. 2 Effect of quality of protein in the maternal diet on the growth of the progeny. Open squares, mean body weights of 9 litters. Hatched squares, mean body weights of 8 litters. Solid squares, mean body weights of 3 litters.

TABLE 5

Effect of Alpha Protein [1] with and without added methionine in the maternal diet on growth of offspring

Maternal diet	No. of litters	No. of pups born alive	Mean body wt on day after birth	No. of each sex at age 3 weeks	Mean body wt		
					At age 3 weeks	At age 14 weeks	At age 26 weeks
			g		*g*	*g*	*g*
Formulated diet [2] with 20% Alpha Protein and 0.35% methionine (ad libitum)	6	65	6.4 ± 0.05 [3]	24 males	39 ± 0.32	380 ± 4.9(6) [4]	450 ± 5.0(6)
				24 females	35 ± 0.3	230 ± 3.0(6)	342 ± 4.4(6)
Formulated diet with 20% Alpha Protein (ad libitum)	15	135	4.4 ± 0.06	16 males	21 ± 0.3	265 ± 4.5(5) [5]	315 ± 4.6(5)
				24 females	18 ± 0.2	186 ± 2.4(15) [5]	212 ± 3.4(15)

[1] Obtained from Nutritional Biochemicals Corporation, Cleveland.
[2] See text for composition.
[3] Mean ± SE of mean.
[4] Number of deaths occurring after age 3 weeks; one randomly selected animal from each litter retained after age 4 weeks.
[5] Numbers were reduced by mortality to those shown after the age of 3 weeks.

take showed that all 3 groups had essentially the same caloric intake. The growth of the male progeny (fig. 2) gives further support to the hypothesis that protein is, with respect to the future growth of the progeny, a crucial dietary component.

With this extremely poor protein only three of the nine pregnant mothers produced viable young, and in view of the considerable spillage of food seen with the experimental animals the estimation of food consumed could have been inaccurate. We therefore examined the effect of Alpha Protein [6] which is known to be deficient in methionine but which represents a dietary stress less severe than that of zein. Experimental animals were fed the formulated diet with 20% Alpha Protein substituted for casein; control animals received the same diet except that 0.35% methionine was added. The average intake during gestation was 186 g/100 g body weight for the experimental animals and 150 g/100 g body weight for the controls. The growth-stunting of the experimental progeny (table 5) was even more severe than that observed following overall dietary restriction. General experience, including our own, has shown that when a diet is deficient in vitamins or protein, the food intake of nonpregnant rats is reduced. However in none of the experiments reported here was food intake reduced under similar dietary conditions and we must conclude that pregnant rats represent an exception to the general rule.

It is well-known that protein intake and protein reserve during mating, gestation and lactation are of great importance. A number of workers including Nelson and Evans (5), Guilbert and Gross (6), and Macomber (7) have altered the level of good-quality protein in otherwise adequate diets before and during reproduction. When the diet contained 5% or less protein, estrus frequently ceased; such a diet after conception resulted in resorption or stillbirth. At somewhat higher levels of protein intake, live young were produced but the weaning weight was subnormal. None of these investigators, however, reported the progress of the offspring after weaning.

CONCLUSION

Both the quality and quantity of protein in the diet of rats during gestation and lactation have profound influence on the subsequent growth of the offspring. The maternal caloric intake probably has subsidiary importance because it determines the amount of protein diverted for use as a source of energy. It is unlikely that deficit of the known minerals and vitamins has played any substantial part in causing the effects of restriction observed in this laboratory.

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[6] Obtained from Nutritional Biochemicals Corporation, Cleveland.

Growth Inhibition of *Dermestes maculatus* by Phytosterols

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ABSTRACT The effect of a β-sitosterol preparation, stigmasterol and ergosterol on the larvae of the zoophagous beetle *Dermestes maculatus* was studied. When the diet contained optimal amounts of cholesterol, an essential nutrient for the larvae, the phytosterols partially inhibited growth and delayed pupation. The cholesterol content of the larvae was also depressed. Stigmasterol had the strongest growth-depressing effect, whereas the sitosterol preparation depressed larval cholesterol to the greatest extent. In the absence of dietary cholesterol, sitosterol (but not stigmasterol nor ergosterol) was able to support partial growth and to induce pupation. The inhibitory action of phytosterols on the development of *D. maculatus* is interpreted as due to interference with the uptake of cholesterol by the larvae. It appears to be analogous to the anti-cholesterolemic action of plant sterols in mammals and birds.

Zoophagous insects have a strict requirement for cholesterol or some of its closely related derivatives. Studies with *Dermestes maculatus* (1) have shown that among a number of derivatives of cholesterol, only 7-dehydrocholesterol is able to induce complete pupation in the larvae. Similar results had been obtained earlier by Clark and Bloch (2) with *Dermestes vulpinus*. In the latter case, β-sitosterol, the most common sterol of higher plants, was reported to lack growth-supporting activity, but when cholesterol was supplied in suboptimal amounts, the plant sterol exhibited considerable sparing action with respect to cholesterol, i.e., the larvae pupated, though no pupation occurred when the same low amount of cholesterol was supplied without additional sitosterol (2, 3).

The present study was prompted by the well-known anti-cholesterolemic action of plant sterols, particularly β-sitosterol, in mammals and birds (4–6), an effect which is generally believed to result from the interference of the phytosterols with the normal absorption of cholesterol from the intestine (7, 8). It was reasoned that a similar interference with the utilization of dietary cholesterol in carnivorous insects might result in impaired development.

This report therefore describes the effect of plant sterols on the utilization of cholesterol by the larvae of *D. maculatus*, as expressed by growth and pupation. An attempt was also made to measure the cholesterol content of the larvae.

MATERIALS AND METHODS

Stock culture of Dermestes maculatus. Larvae of *D. maculatus* were reared at 30° with a diet of fish meal enriched with 15% dried yeast [1] and containing a chunk of hog adipose tissue. All the following steps were also carried out at 30°.

Egg collection and incubation. Adult beetles were transferred to a fresh stock diet. Eggs were collected after 2 days by removing the beetles and sieving the diet. The eggs were placed on moistened filter paper in covered Petri dishes. The larvae hatched after 2 days.

Growth experiments. Two newly hatched larvae, not over 3 hours old, were placed in small cloth-covered plastic vials containing 3 g of the experimental diet. The vials were kept at 30° in an incubator in which a moist atmosphere was maintained by open beakers containing water. Each dietary treatment comprised 20 larvae distributed into 10 vials. The larvae in each vial were weighed together at intervals, the average weight per larva was calculated for each vial, and the figures

Received for publication August 19. 1966.

[1] Hopkin and Williams Ltd., Chadwell Heath, Essex, England.

obtained within each treatment were averaged. After about 40 days' growth, the contents of the vials corresponding to each dietary treatment were pooled and pupation was examined every 2 to 4 days.

Diets. In the first experiment, the basal diet described by Levinson (9) was used (diet 1). This was subsequently modified by replacing the mixture of B-vitamins by yeast extract, as follows: casein, vitamin and fat-free,[2] 41.8; starch, soluble, 25.0; fructose,[3] 16.7; salt mixture BS 2407,[4] 2.6; yeast extract, dry 5.0; and distilled water, 9.0. The casein, starch, fructose and salt were mixed, and the yeast extract, dissolved in water, was added and the mixture was stirred until homogeneous. Sterol and oil supplements were added as solutions in ethyl ether. The solvent was allowed to evaporate at room temperature and the mixture was again stirred thoroughly.

Sterols. Cholesterol USP;[5] β-sitosterol, ergosterol and stigmasterol (purum):[6] sterols were found to be pure by gas-liquid chromatography, except β-sitosterol, which was a mixture of campesterol and β-sitosterol in the ratio of 4 to 6, and which will therefore be designated "β-sitosterol preparation," or simply "sitosterol," to avoid confusion with the pure compound.

Determination of sterols in insect larvae. The pooled larvae from each dietary treatment were dried at 100° and ground to a powder. This was refluxed for one-half hour with a mixture of 20 ml ethanol and 2 ml aqueous potassium hydroxide 50% (w/w). The unsaponifiable matter was recovered by extraction with petroleum ether. After washing and drying of the extract, the solvent was removed and the sterols were converted to trimethylsilyl ethers by direct reaction of the unsaponifiable matter with the silylating mixture. The latter was composed of N, N-dimethylformamide,[7] hexamethyldisilazane (purum)[8] and trimethylchlorosilane[9] in the proportions 40:40:1 (10). After standing for 30 minutes an aliquot was injected into the gas chromatograph. The instrument was a Perkin-Elmer 801 gas chromatograph fitted with a flame ionization detector. Separation of the sterols was effected on a 183-cm glass column, 0.6-cm outer diameter, packed with 2% SE-30 on silanized Gas-Chrom P, 100/120.[10] Column temperature was 235° and pure nitrogen was used as the carrier gas. The separations obtained were similar to those reported by Rozanski (11). Cholesterol, stigmasterol and ergosterol were identified and their concentrations calculated by comparison with reference standards, but since no standards for β-sitosterol and campesterol were available, peak areas were used to express relative concentrations, whereas identification of the peaks was readily achieved by the relative retention times, as published in the literature (11).

RESULTS

In the first trial, the basal lipid-free diet (no 1) was used for studying the growth effect of 0.2% cholesterol in the presence of various concentrations of sitosterol. The results obtained are presented in table 1.

TABLE 1

Effect of cholesterol and sitosterol[1] on growth of D. maculatus (diet 1)

Sterol added	Mean wt/larva at 40 days
	mg
None	—[2]
0.2% Cholesterol	14.43
0.2% Cholesterol + 0.2% sitosterol	7.28
0.2% Cholesterol + 0.4% sitosterol	7.08
0.2% Cholesterol + 0.6% sitosterol	6.80
0.2% Sitosterol	5.10
0.4% Sitosterol	4.11
0.6% Sitosterol	4.65

[1] A mixture of 40% campesterol and 60% β-sitosterol.
[2] No larvae survived.

The inhibitory effect of the plant sterol on larval growth was very marked and became more pronounced as the sitosterol level was increased from 0.2 to 0.6%. When no cholesterol was present, the same 3 levels of sitosterol supported par-

[2] Casein and starch obtained from British Drug Houses Ltd., Poole, England.
[3] Fructose and yeast obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] Obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, England.
[5] See footnote 3.
[6] Obtained from Fluka AG., Buchs SG., Switzerland.
[7] See footnote 2.
[8] See footnote 6.
[9] Applied Science Laboratories, Inc., State College, Pennsylvania.
[10] See footnote 9.

tial growth and prevented the mortality of the larvae that continued to receive the basal sterol-free diet.

Subsequently, it was found that the inclusion of about 1% oil in the diet improved larval development considerably (table 2).

Addition of olive oil to the diet increased the weight of the larvae and shortened the time needed to reach the pupal stage. All subsequent tests were therefore carried out with the modified diet (no. 2) described under Materials and Methods, containing 1% olive oil.

The effect on growth of 3 plant sterols was investigated next. Results obtained after 40 days' growth are presented in table 3.

All 3 sterols inhibited growth, the effect being strongest for stigmasterol, weaker for ergosterol and least pronounced for the β-sitosterol preparation. A mixture of 0.1% cholesterol and 0.3% sitosterol produced nearly the same growth as the same mixture at double the concentration (0.2 and 0.6% for cholesterol and sitosterol, respectively). An additional point of interest is the effect exerted by these 3 phytosterols in the absence of dietary cholesterol. As in the first trial, the sitosterol preparation exhibited some growth-promoting properties, the same effect being obtained at dietary levels of 0.3 and 0.6%. Stigmasterol did not support growth, but the larvae remained alive until the end of the 40 days' experiment, whereas ergosterol neither supported growth nor prevented mortality.

Additional data on growth inhibition caused by sitosterol, stigmasterol and ergosterol are presented in table 4.

Stigmasterol again proved to be the most effective inhibitor, whereas sitosterol and ergosterol produced weaker and closely similar effects. The growth-promoting effect of sitosterol in the absence of dietary cholesterol was confirmed. Also, stigmasterol again lacked growth-supporting activity, but gave improved survival. Ergosterol produced even higher mortality than the sterol-free basal diet. When the plant sterols were administered in various combinations, growth inhibition varied. The mixture of 0.3% sitosterol and 0.3% ergosterol depressed growth more than did 0.6% of either of these sterols (synergistic effect). Conversely, an antagonistic effect was observed for sitosterol and stigmasterol which, in combination, lost their inhibitory action.

The cholesterol content of the larvae (table 4) reflects, but does not parallel, growth inhibition by plant sterols. Thus, all phytosterols tested decreased the amount of cholesterol per larva, but sitosterol exhibited the strongest cholesterol-depressing effect, whereas stigmasterol was the most effective growth inhibitor. Also, in the absence of dietary cholesterol, only very little cholesterol was observed in the larvae, even though some growth occurred, as with sitosterol alone. Generally, larval cholesterol is more sensitive to dietary influences than larval growth.

TABLE 2

Effect of olive oil on growth and pupation of D. maculatus (diet 2)

Addition to diet	Mean wt/ larva at 39 days	Avg time to pupation
	mg	*days*
0.05% Cholesterol	7.24	70.3
0.05% Cholesterol +1% olive oil	15.71	56.5
0.05% Cholesterol +2% olive oil	18.55	52.5
0.2% Cholesterol	8.08	72.1
0.2% Cholesterol +2% olive oil	18.82	52.3

TABLE 3

Effect of sterols on growth of D. maculatus (diet 2 with 1% olive oil)

Sterol added	Mean wt/ larva at 40 days
	mg
None	— [1]
0.1% Cholesterol	17.08
0.2% Cholesterol	24.18
0.1% Cholesterol + 0.3% sitosterol [2]	14.79
0.1% Cholesterol + 0.3% ergosterol	13.08
0.1% Cholesterol + 0.3% stigmasterol	9.44
0.2% Cholesterol + 0.6% sitosterol [2]	14.08
0.3% Sitosterol [2]	9.16
0.3% Ergosterol	— [3]
0.3% Stigmasterol	1.26
0.6% Sitosterol [2]	9.27

[1] Only 2 larvae survived.
[2] A mixture of 40% campesterol and 60% β-sitosterol.
[3] Only 3 larvae survived.

TABLE 4

Effect of dietary sterols on growth and sterol content of D. maculatus (diet 2, with 1% olive oil)

Sterol added	Mean wt/ larva at 38 days	Cholesterol content		Phytosterols/larva		
				β-Sitosterol	Campesterol	Stigmasterol
	mg	*μg/larva*	*μg/mg dry wt*	*μg*	*μg*	*μg*
None	1.59	0.94	2.04	—	—	—
0.2% Cholesterol	17.67	35.6	5.50	—	—	—
0.2% Cholesterol + 0.6% sitosterol [1]	9.42	8.3	2.79	1.3	2.3	—
0.2% Cholesterol + 0.6% ergosterol	9.75	16.6	5.43	—	—	—
0.2% Cholesterol + 0.6% stigmasterol	7.00	10.5	4.88	0.6	—	0.9
0.2% Cholesterol + 0.3% sitosterol [1] + 0.3% ergosterol	6.81	10.2	4.76	0.8	1.0	—
0.2% Cholesterol + 0.3% sitosterol [1] + 0.3% stigmasterol	16.59	18.3	2.99	1.5	2.5	0.7
0.2% Cholesterol + 0.3% ergosterol + 0.3% stigmasterol	8.94	18.4	6.38	—	—	0.2
0.2% Cholesterol + 0.2% sitosterol [1] + 0.2% ergosterol + 0.2% stigmasterol	8.79	12.9	4.70	2.8	1.6	0.2
0.6% Sitosterol [1]	6.59	0.9	0.42	3.4	2.7	—
0.6% Ergosterol	— [2]	—	—	—	—	—
0.6% Stigmasterol	1.48	0.6	1.29	1.7	—	0.2

[1] A mixture of 40% campesterol and 60% β-sitosterol.
[2] No larvae survived.

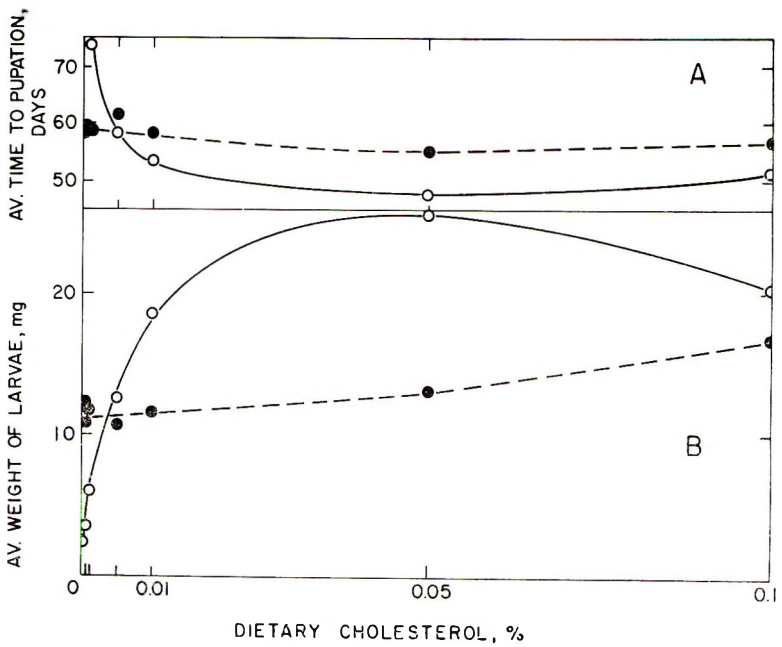

Fig. 1 Effect of increasing cholesterol levels on development of *D. maculatus* in the presence of 0.3% sitosterol (●) and in the absence of sitosterol (O). Average time to pupation is shown in the upper part (A) and growth response at 40 days is given in the lower part (B) of the figure. Pupation became deficient at cholesterol levels below 0.005%.

In view of the two opposing effects produced by sitosterol, i.e., partial growth support in the absence of dietary cholesterol and growth inhibition in the presence of near-optimal doses of cholesterol, a trial was carried out to study the effect on larval growth and pupation of increasing amounts of cholesterol, in the presence, as well as in the absence of additional sitosterol. The results of this trial are presented graphically in figure 1.

Two curves representing growth as a function of cholesterol concentration were obtained, according to whether sitosterol was present or not. The growth curve obtained without additional sitosterol rapidly rises with cholesterol concentration, up to an optimal level of 0.05% cholesterol. The presence of 0.3% sitosterol in the diet, however, completely changes the shape of the curve: growth is almost completely independent of the dietary cholesterol concentration, increasing only slightly at higher cholesterol levels. The 2 curves intersect each other at a point corresponding to 0.004% cholesterol. Below this concentration, the addition of sitosterol causes improved growth, but above this cholesterol level, dietary sitosterol causes growth inhibition. Under the conditions of the test, growth inhibition by sitosterol is maximal when the dietary cholesterol level is about 0.05%.

The picture is very similar when pupation is considered (fig. 1). In the presence of 0.3% sitosterol, the amount of dietary cholesterol had no influence on the average age of pupating larvae: at low cholesterol concentrations, or in the absence of dietary cholesterol, sitosterol alone was able to induce pupation, whereas at near-optimal cholesterol concentrations, the effect of sitosterol was to delay pupation.

DISCUSSION

The results presented in this study leave no doubt as to the inhibitory effect on larval development produced by the sitosterol preparation, stigmasterol and ergosterol, in the presence of near-optimal amounts of cholesterol. The effect is noted mainly from the decreased larval growth, but pupation was also somewhat delayed.

The data on the sterol content of the larvae provide a possible explanation for the effects observed. It is clear that the plant sterols cause a decrease in the cholesterol level per larva and, at least in the case of sitosterol, the tissue cholesterol is also depressed on a dry-weight basis. Since cholesterol is an essential nutrient for these larvae, it appears that the plant sterols inhibit larval development by interfering with the uptake of cholesterol by the insects.

However, there is no direct relation between body cholesterol and growth, as seen from the relative effects of sitosterol and stigmasterol in tables 3 and 4. Also, when several plant sterols are supplied together, unexpected effects on growth show up. Thus, the sitosterol preparation and ergosterol act synergistically with respect to growth inhibition, whereas sitosterol and stigmasterol are antagonists and lose most of their growth-depressing activity when administered together. No such effects are observed with a mixture of ergosterol and stigmasterol. These effects are not reflected by the larval cholesterol levels. Furthermore, when no dietary cholesterol is supplied, the cholesterol content per larva drops to very low levels, though growth may be appreciable, as for diets containing sitosterol, for instance.

The biological action of the sitosterol preparation deserves special comment: although this material does not cause the strongest growth inhibition, it has the greatest cholesterol-depressing effect. Furthermore, sitosterol produces appreciable growth and good pupation in the absence of dietary cholesterol, unlike stigmasterol and ergosterol. Where gas-chromatographic data were obtained, β-sitosterol was noted in appreciable amounts in the larvae; stigmasterol and ergosterol were deposited to a very slight extent only, nor did these sterols support growth. It is concluded that sitosterol can, to some extent, replace cholesterol with respect to growth and pupation.

The commercial sitosterol used in these tests contained a large proportion of campesterol. Therefore it has not been possible to ascertain to what extent β-sitosterol and campesterol contribute to the

inhibition of growth, depression of larval cholesterol level and cholesterol-replacing action exhibited by the commercial sitosterol.

The apparent contradiction between the cholesterol-replacing action of sitosterol and its interference with cholesterol utilization, when the latter sterol is supplied at near-optimal levels, is resolved by consideration of the results of the last trial (fig. 1). In the absence of dietary sitosterol, cholesterol produces the expected effect on larval growth and pupation. However, in the presence of 0.3% sitosterol, the effect of increasing cholesterol concentrations is cancelled, and nearly constant growth and pupation times are obtained throughout the range of cholesterol concentrations, except at the highest concentration of 0.1%, where growth is slightly increased. It appears that a sitosterol level of 0.3% effectively blocks cholesterol utilization, and larval development observed under these conditions is essentially brought about by the plant sterol preparation, and is independent of the dietary cholesterol concentration. As a result, the 2 curves representing growth vs. cholesterol level, with and without additional sitosterol, intersect each other at a point corresponding to 0.004% cholesterol. The situation is similar with respect to the average age of pupating larvae. At cholesterol levels below 0.004%, additional sitosterol will improve growth and pupation, thus exhibiting cholesterol-replacing or sparing activity. But at higher cholesterol levels, utilization of cholesterol would be impaired by supplemental sitosterol, and the result is seen as depressed growth and delayed pupation.

The present data on the promotion of growth and pupation of *D. maculatus* by the sitosterol preparation, used as the sole source of dietary sterol, are at variance with the observations of Clark and Bloch (2) and Clayton and Bloch (3) on the cholesterol-sparing action of β-sitosterol in *Dermestes vulpinus*. These authors reported that when the diet was devoid of cholesterol, sitosterol was unable to support growth and induce pupation. Although the presence of traces of cholesterol in the diet cannot be excluded in our experiments, it appears more likely that the appreciable effect on growth and pupation found for sitosterol in the absence of cholesterol may be the result of the addition of olive oil to the diet and reflects the requirement of the larvae for essential fatty acids (the olive oil contained about 17% linoleic acid). This point will be investigated further.

The interference of plant sterols with the utilization of cholesterol by *D. maculatus* is analogous to the prevention, by phytosterols, of the hypercholesterolemia caused by high dietary levels of cholesterol in mammals and birds (8). It is tempting to assume that the underlying mechanism of action is the same in all cases.

According to Davis (12) β-sitosterol and cholesterol form a complex of low solubility or dispersability in bile salt solution. This provides a possible explanation for the interference of plant sterols with cholesterol absorption. Glover and Green (13) have shown that rapid cholesterol absorption takes place by an exchange and transfer between mucosal lipoproteins. These authors assume that sitosterol could occupy active sites on such lipoproteins, thus causing a block in cholesterol absorption. Whatever the mechanism of action, *Dermestes* could possibly be used as a test organism for qualitative studies on the anti-cholesterolemic activity of plant sterols, with the advantage of requiring only relatively small amounts of test substances.

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Comparison of the Actions of Vitamins D_2 and D_3 in the Chick with their Retention in Serum, Liver and Intestinal Mucosa [1]

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ABSTRACT The response of rachitic chicks to single doses of vitamins D_2 and D_3 was evaluated, 18 hours after dosing, in terms of: (a) the concentration of vitamin D in intestinal mucosa, blood serum and liver, (b) the response of serum calcium and inorganic phosphorus, and (c) the calcium transport by the ileum incubated in vitro in a low Na medium. The vitamin activity, biologically assayed using the calcium transport by the rat duodenum in vitro as a criterion, was similar for both sterols in the intestinal mucosa, but was 3 to 4 times higher in the serum and liver of the chicks dosed with vitamin D_3 than of those given vitamin D_2. Both sterols lowered the serum inorganic phosphorus of the chick to a similar extent, but vitamin D_3 was more effective in increasing serum calcium. Vitamin D_3 was over 10 times as effective as vitamin D_2 in promoting the in vitro calcium transport by the chick ileum. It is suggested that the lower biological activity of vitamin D_2 than of vitamin D_3 in the chick is due to reduced physiological effectiveness of the former in the intestinal mucosa and either poorer absorption or more rapid catabolism of the inactive sterol.

The antirachitic efficacy of vitamin D_2 in chicks is considerably lower than that of vitamin D_3 but there are large discrepancies among various estimates of the activity ratios. Early estimations of vitamin D_3-to-vitamin D_2 efficacy ratios varied between 15 to 200 (1–6), whereas more recent observations (7, 8) suggest a ratio of only 8 to 11. The difference of potency between the 2 forms of vitamin D, despite their chemical similarity, is of theoretical interest with respect to the mode of action of vitamin D.

In the present study we investigated two specific possibilities: (a) whether vitamin D_2 accumulated to a lesser degree in the tissues of the chick than did vitamin D_3 due either to impaired absorption or increased rate of catabolism, or (b) whether at comparable tissue concentrations in the chick, vitamin D_2 was physiologically less active than vitamin D_3.

EXPERIMENTAL AND RESULTS

The retention of administered vitamin D_2 and D_3 in the serum, intestinal mucosa, and liver of rachitic chicks was determined 18 hours after a single dose of the steroid. One-day-old White Leghorn chicks were obtained from a commercial hatchery and placed immediately in a battery-brooder and fed the vitamin D-deficient diet shown in table 1. Eighteen days later they were divided into 5 groups of 12 chicks each. Groups 1 and 2 received 2000 IU of crystalline vitamin D_2 or D_3, respectively; groups 3 and 4 received 10,000 IU of crystalline vitamin D_2 or D_3, respectively; group 5 was the control group and was given only the solvent. The D-vitamins were dissolved in propylene glycol and given by crop-intubation. Eighteen hours after dosing, blood samples were obtained by cardiac puncture and the birds were anesthesized by an intracardiac dose of sodium pentobarbital. The liver and the entire small intestine were removed and placed on ice. After thorough washing out of the intestinal contents with saline, the intestinal mucosa was obtained by slitting the entire length of the small intestine and scraping off the mucosa with a glass slide. All samples were frozen immediately. Serum samples were separated from the blood and frozen immediately after separation.

Received for publication August 29, 1966.

[1] Supported in part by Public Health Service Research Grant no. AM-00668 from the National Institutes of Health.

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TABLE 1

Composition of the experimental vitamin D-depletion chick diet

	%
Soybean meal (50% protein)	45.00
Glucose	47.00
Peanut oil, refined	3.00
Choline chloride	0.10
DL-Methionine	0.15
Vitamin mixture [1]	0.25
Salt mixture [2]	0.50
Potassium phosphate, monobasic	1.00
Sodium phosphate, monobasic	1.00
Calcium carbonate, precipitated	2.00

[1] Supplied per kg of ration: (in milligrams) thiamine, 25; riboflavin, 16; Ca pantothenate, 20; pyridoxine, 6; biotin, 0.6; folic acid, 4; menadione, 2; niacin, 150; ascorbic acid, 250; inositol, 100; vitamin B_{12}, 0.02 mg; and vitamin A, 10,000 IU; and α-tocopherol, 5 IU.

[2] Supplied in mg/kg ration: NaCl, 2,877; $FeSO_4$, 286; $MgSO_4$, 1,000; $MgCO_3$, 500; $MnSO_4$, H_2O, 150; KI, 6; $CuSO_4$, 8; $ZnCO_3$, 150; and $Na_2MoO_4 \cdot 2H_2O$, 0.6.

TABLE 2

Composition of rat rachitogenic diet [1]

	%
Casein	17.00
Brewer's yeast	4.70
Peanut oil, refined	7.50
Salt mixture [2]	1.00
Calcium carbonate	3.75
Cornstarch	42.55
Glucose	23.50

[1] Each kilogram of diet was supplemented with 10,000 IU of vitamin A.

[2] Supplied in mg/kg ration: NaCl, 2,877; $FeSO_4$, 100; $MgSO_4$, 64; KCl, 448; $FeSO_4$, 94; KI, 0.32; $MnSO_4 \cdot H_2O$, 1.57; and $CuSO_4$, 0.36.

Calcium was determined in the serum by the method of Harrison and Harrison (9) and inorganic phosphate by the method of Fiske and Subbarow (10). For determination of vitamin D activity, samples from the chicks that received 2000 IU were used. Equal amounts of serum or tissue from 2 chicks were pooled for each assay. Intestinal mucosal samples were diluted with a measured quantity of water and sonicated at 0°. Liver samples were homogenized in a Waring Blendor and made up to a constant total volume. The tissue homogenates were kept frozen until the assay was performed.

Vitamin D activity was biologically assayed in rats of the Sprague-Dawley strain that had been fed a rachitogenic diet (table 2) for 3 weeks starting at weaning. A measured aliquot of serum or tissue homogenate was administered by stomach tube to 2 rats. Control rats received graded doses of vitamin D_2 in propylene glycol. Seventy-two hours after dosing, the rats were anesthetized by intraperitoneal sodium pentobarbital, bled by aortic puncture and the first 12.5 cm of the small intestine was taken for an in vitro measurement of concentrative calcium transport by the everted intestinal loop method (11). The response criterion was the serosal-to-mucosal ratio of calcium (C_s/C_m) developed after incubation. Figure 1 shows two standard curves for the dose response to vitamin D in terms of concentrative transport of calcium by rat duodenum in vitro. Concentrative transport of calcium as measured by C_s/C_m is linearly related to the log of the vitamin D dosage. The dose response curve showed some variation when determined at different times and for this reason each assay was calculated on the basis of the standard curve determined simultaneously with the tissue samples. An additional criterion of vitamin D

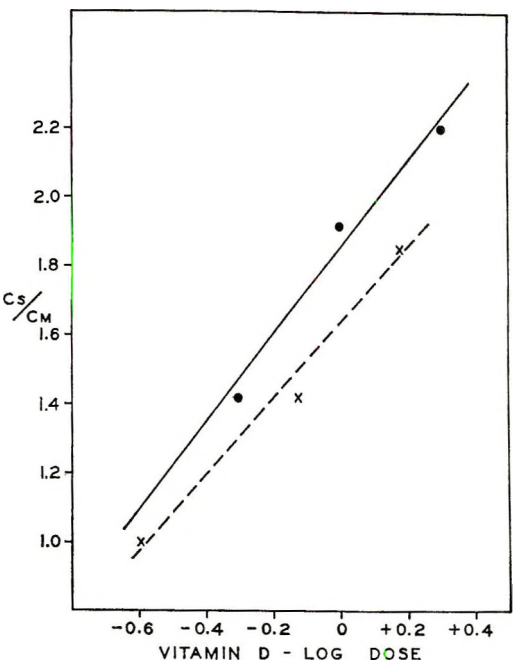

Fig. 1 Vitamin D dose response curves: serosal-to-mucosal (C_s/C_m) ratios of calcium of everted rat duodenal loops as a function of vitamin D_2 dosing of the rat 72 hours prior to the assay. Each point represents the average of at least 3 duodenal preparations. Two different assays are given indicating variations in responses observed over a period of several months.

activity was the increase of serum inorganic phosphate concentration of the rats.

The concentrations of serum calcium and inorganic phosphorus of the rachitic chicks 18 hours after a single dose of vitamins D_2 and D_3 are shown in figure 2. Plasma inorganic phosphorus concentrations decreased following vitamin D administration, and there was little difference between the response to vitamin D_2 or vitamin D_3. Both steroids caused an increase in serum calcium concentration but there was a much greater response to vitamin D_3 than to equal amounts of vitamin D_2. These results are in agreement with the observations of Chen and Bosman (7).

The estimated vitamin D activities in chick serum, liver and intestinal mucosa are shown in table 3. No vitamin D activity could be detected in tissues from the rachitic chicks. The assayed vitamin D activity in intestinal mucosa was approximately the same for chicks that had received either vitamin D_2 or vitamin D_3. However, the vitamin D activity was about 3 times higher in the serum of birds that had received vitamin D_3 than in that from birds that had received vitamin D_2. Similarly, the activity in the livers was also

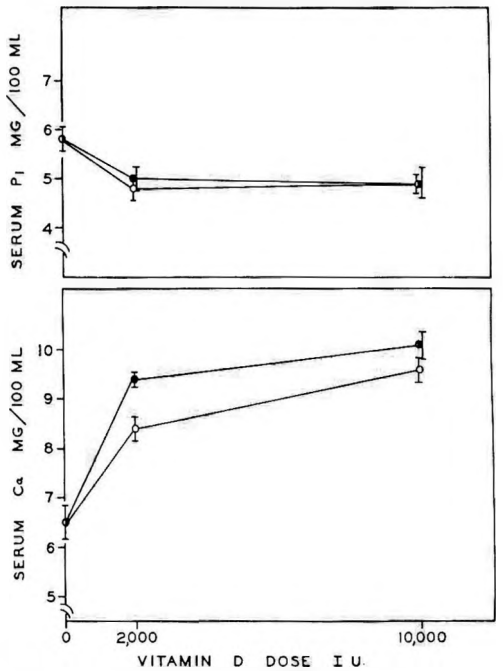

Fig. 2 The response of serum calcium and inorganic phosphorus in the chick to either vitamin D_2 (○) or vitamin D_3 (●) given as a single dose. The vertical lines denote the standard error of the mean.

TABLE 3

Estimated vitamin D activity in tissues of chicks 18 hours after administration of 2000 IU vitamin D_2 or D_3

Treatment	Plasma		Total liver	Total intestinal mucosa
	IU/ml	*IU/10 ml* [1]	*IU*	*IU*
Rachitic	0	0	0	0
Vitamin D_2-treated	4.4	44	40	154
Vitamin D_3-treated	15.5	155	120	184

[1] This is an estimate of the total plasma volume and indicates the total vitamin D activity in circulating plasma.

TABLE 4

Response to vitamin D_2 and vitamin D_3 as measured by concentrative calcium transport by chick ileum [1]

Treatment	C_s/C_m	*Serum Ca*
		mg/100 ml
Rachitic-untreated	0.77 ± 0.04 [2]	7.1 ± 0.3
Vitamin D_2, 100 IU	0.98 ± 0.10	6.8 ± 0.6
Vitamin D_2, 500 IU	1.27 ± 0.14	6.6 ± 0.2
Vitamin D_3, 10 IU	1.00 ± 0.08	6.8 ± 0.2
Vitamin D_3, 50 IU	1.42 ± 0.14	8.9 ± 0.5

[1] The vitamins were administered 18 hours before the experiment by crop intubation.
[2] Each value represents the mean of intestinal preparations from 5 chicks ± standard error of mean.

about 3 times as high in the vitamin D_3-treated birds.

In another series of experiments, the efficacy of vitamins D_2 and D_3 fed to rachitic chicks was compared using the calcium transport system of the everted chick intestine in vitro. The birds in this study were obtained and maintained as in the previous experiment. Four lots of 5 birds were given 100 and 500 units of vitamin D_2 and 10 and 50 units of vitamin D_3, respectively. As before, the vitamins were dissolved in propylene glycol and administered by crop intubation. Eighteen hours after vitamin D administration the birds were bled by heart puncture and calcium was determined in the serum. The ileum was removed, everted and incubated in vitro in a medium containing: (in mmoles /liter) mannitol, 198; KCl, 25; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgSO_4$, 0.85; $CaCl_2$, 0.25; and glucose, 20. The buffer also contained 2.5 μCi of ^{45}Ca/100 ml. The incubation was carried out for 90 minutes at 37° and the final serosal-to-mucosal ^{45}Ca concentration ratio (C_s/C_m) was taken as the criterion for calcium transport. The system had been found previously to be very sensitive to vitamin D treatment.[3]

The results of this experiment are presented in table 4. There was only a slight effect of vitamin D_2 on the concentration of serum calcium of the rachitic chick even when given at a level of 500 IU, and the change was a reduction of the concentration of serum calcium. Vitamin D_3, however, increased the serum calcium concentration at a dose level of 50 units; 10 units did not significantly alter the serum calcium concentration. The system which appeared to be most sensitive to vitamin D was the concentrative calcium transport of everted intestinal loops in vitro in low sodium buffer, as measured by the C_s/C_m ratio. The increase in the C_s/C_m ratios was directly related to the quantity of vitamin D given. Fifty units of vitamin D_3 gave a higher C_s/C_m ratio than 500 IU of vitamin D_2, indicating that the efficacy ratio may be even greater than 10:1 as suggested by Chen and Bosman (7).

DISCUSSION

The biological assay for vitamin D activity, using the in vitro mucosal-serosal calcium transport by rat duodenum, proved to be sensitive to very small amounts of vitamin D in agreement with the results of Dowdle et al. (12). However, when standard curves were determined at different times, there were considerable differences both in the basal values, as well as in the response to vitamin D. The values determined here should, therefore, be considered to be approximations only. However, in any one assay, tissues of chicks receiving each of the vitamin D treatments were represented, and therefore the results are valid for comparison between the activity of the 2 forms of vitamin D in each tissue.

Both vitamins D_2 and D_3 increase the activity of the concentrative transport system for calcium in the chick ileum as demonstrated by in vitro incubation in a low sodium buffer. The efficacy ratio of vitamin D_2 to vitamin D_3 as determined by this method is perhaps somewhat higher than the 11: 1 value given by Chen and Bosman (7) but is closer to this value than to the highest ratios reported in earlier studies.

The results of the assay demonstrate that vitamin D_2 was incorporated into the intestinal mucosa and absorbed by the chick to a greater extent than indicated by its relative physiological activity when compared with vitamin D_3. Although there was a several-fold difference between the assayed concentrations of vitamins D_2 and D_3 in the serum and liver, the concentrations were approximately the same in the intestinal mucosa. Schachter et al. (13) have shown previously that vitamin D has to be present in the intestine in order to exert its effect. However, in the rat there is a period of about 4 hours between the introduction of the vitamin into the intestine and the calcium transport response. During this period the vitamin may be converted to an active form by metabolic alteration of the molecule or by complexing with an acceptor molecule or it may induce synthesis of a protein fraction which is important in calcium transport (14). Although vitamin D_2 was present in the intestinal mucosa of the chick in concentrations comparable to those of vitamin D_3 its physiologic effectiveness was much

[3] Hurwitz, S., H. C. Harrison and H. E. Harrison, unpublished data, 1966.

less than that of vitamin D_3. Presumably the intestinal mucosal cell of the chicks cannot activate the vitamin D_2 molecule as effectively as that of vitamin D_3. The differences between the concentrations of vitamins D_2 and D_3 in the serum and liver of the chick may represent more effective absorption of the "active" molecule or more rapid catabolism of the inactive form. The present experiment does not establish this point.

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Methods of Comparing Protein Quality of Soybean Infant Formulas in the Rat

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ABSTRACT Protein quality and caloric efficiency value of 4 liquid soybean formulas were compared in the rat. Protein efficiency ratios (PER) were measured using dry diets containing 10% protein formulated from the lyophilized soybean formula products, and liquid diets made from the formulas diluted with carbohydrate to provide a level of protein equivalent to that provided in the dry diets. PER for the individual formulas were comparable when fed either as dry (lyophilized) or liquid diets. Protein quality of the dry diets, as percentages of the PER attained with the standard casein were 97, 80, 66 and 62% for formulas A, B, C and D, respectively. Caloric efficiency value was evaluated by feeding the concentrated formulas as the sole article of the diet. When fed as concentrated formulas, growth of the rats receiving formulas A, B, and C were similar, although caloric efficiency values were significantly higher with formula A than with the other formulas. Caloric efficiency values provided better measure of protein quality than did overall weight gains. Growth of the animals fed concentrated formula D as the sole article of the diet was poor and caloric efficiency values were low.

Techniques for the measurement of protein quality and nutritive value of liquid formulas usually require lyophilization and incorporation of the material into dry diets. We have been interested in methods for measurement of protein quality of soybean infant formulas[1] fed as liquids, because the development of formulas containing isolated soybean protein in place of soybean flours necessitated many measurements of protein quality. In the studies reported here, protein quality of 4 soybean formulas was evaluated after lyophilization, using the protein efficiency ratio. Protein quality of these formulas was also evaluated when fed as liquids, after dilution with carbohydrate to a level of protein equal to that fed in the dry diets. In a third comparison, the formulas were fed as undiluted liquids as the sole article of the diet.

The results demonstrate that protein quality of soybean formulas can be evaluated with liquid diets, as well as with dry diets, thereby circumventing the need for lyophilization. When the formulas were fed as the sole article of the diet, weight gains did not reflect protein quality although caloric efficiency values were decreased with decreasing protein quality.

EXPERIMENTAL

Groups of 10 male weanling rats each, of the Wistar strain, were selected on the basis of body weight and litter. Each animal was housed in an individual screen-bottom cage in an air conditioned animal room, maintained at 23° to 24°. Body weights and food and water consumption were recorded at appropriate intervals during the 4-week tests.

Adequate volumes of each of the concentrated soybean formulas containing 134 kcal/100 ml (table 1) were lyophilized in one-liter bottles containing 150 ml of formula on a multiple manifold, dry ice-methanol freeze-dryer. Each lyophilized formula was analyzed for nitrogen and fat to determine the amount needed in the diet to supply 10% protein (N × 6.25) as shown in table 1. The casein diet and the diets made from soybean formulas A, B and C contained 12% fat from the formula or added soybean oil, or both, whereas the diet from formula D contained 17.1% fat, because of the relatively low protein and high fat content of the for-

Received for publication September 8, 1966.

[1] The formulas studied were ProSobee and Sobee (Mead Johnson and Company, Evansville, Indiana), Mull-Soy (The Borden Company, New York), and Soyalac (Loma Linda, Riverside, California).

TABLE 1
Formula and diet composition

Formula	Protein	Fat	Carbohydrate	Ash
	g/100 ml	*g/100 ml*	*g/100 ml*	*g/100 ml*
Approximate analyses of concentrated soybean formulas				
A	5.0	6.8	13.5	1.0
B	6.4	5.2	15.5	1.3
C	6.0	7.0	10.0	1.5
D	4.1	8.0	12.0	0.3

Composition of dry 10% protein diets	%
Casein or lyophilized soybean formula (to give 10% protein)	
Soy oil (to give 12% fat) [1]	
Vitamin mixture [2]	0.35
Mineral mixture [3]	4.0
Non-nutritive fiber	4.0
Oleum percomorphum	0.015
dl-α-Tocopheryl acetate	0.005
Amidex [4]	to make 100

[1] Including fat supplied by formula. Diet containing soybean formula D contained 17.1% fat, all from the formula.
[2] Sarett and Snipper mixture (12), with ascorbic acid omitted.
[3] Jones and Foster mixture (13), with 10 ppm F added as NaF.
[4] Cornstarch (partially hydrolyzed), Corn Products Company, New York.

mula. The protein efficiency ratio (PER) of these diets was measured using the method described by Longenecker et al. (1). PER was also determined using the liquid soybean formulas, diluted with Dextri-Maltose [2] solution to supply the same levels of protein, as a percentage of calories, as did the dry diets. These were prepared fresh each day and fed in sterilized drinking tubes.[3]

The caloric efficiency value of the formulas was evaluated in 4 additional groups of rats that received the liquid soybean formulas undiluted, i.e., 134 kcal /100 ml, as the sole article of the diets. These were also fed fresh each day.

After 4 weeks, the animals were killed, and selected organs were weighed. The percentage of protein in the plasma was measured according to Kingsley (2). The gastrointestinal tract was discarded and the remainder of the carcass was autoclaved in sealed jars. After homogenization of the carcasses, protein levels (N × 6.25) were determined by a micro-Kjeldahl technique. Lipid in carcass and liver were extracted and measured by the technique of Folch et al. (3). Amino acid composition of the proteins in the soybean formulas was measured using methods similar to those reported previously (1).

RESULTS

Dry diets — protein efficiency ratio. The control animals fed casein had a protein efficiency ratio (PER) of 2.89 g gain/g of protein as shown in table 2. The PER of the soybean formulas ranged from a high of 2.70 to a low of 1.80 g gain/g of protein, with values for soybean formulas A, B, C and D equal to 97, 80, 66 and 62%, respectively, that of casein. Weight gain of the casein control group was 76 g with gains of 79, 59, 44 and 34 g with soybean formulas A, B, C and D, respectively.

Liquid diets — protein efficiency ratio. Weight gains of the animals fed the liquid diets were similar to those attained with the respective dry diets as shown in table 2. Four-week weight gains of 78, 66, 44, and 29 g were achieved with formulas A, B, C and D, respectively. The PER's were also similar to those found with the dry diets. The PER for the animals fed soybean formula A was 2.84 with the liquid diet and 2.79 with the dry diet. The largest difference between liquid and dry diets was in PER values for formula D. The difference of 0.21 g gain/g of protein (dry diet, 1.80 and liquid diet, 1.59) was not statistically significant.

Caloric efficiency value. When the concentrated soybean formulas were fed (undiluted) as the sole article of the diet, weight gains of 186, 183 and 170 g were achieved by the animals fed soybean formulas A, B and C. However, with the relatively low level of protein in formula D (12.3% of calories) and with protein quality equal to 62% of that of casein, weight gains were only 55 g in 4 weeks. The concentrated formulas provided higher protein levels than were fed in the PER diets. Protein supplied 9.6% of the calories in the dry diets compared with 15.2, 17.3 and 18.3% in formulas A, B and C, respectively. At these higher protein levels, weight gain did not show differences in protein quality.

Caloric efficiency value, a measure of the overall utilization of the diet, was significantly higher with formula A (110 g

[2] Dextri-Maltose, Mead Johnson and Company.
[3] Glass watering device — LC-273, Wahmann Manufacturing Company, Baltimore, Maryland.

TABLE 2

Four-week data from male weanling rats fed dry or liquid diets for measurement of protein efficiency ratios or concentrated liquid diets for measurement of caloric efficiency

Protein source	Protein level, % diet		4-week data						
					Protein efficiency				
			Wt gain	Protein intake	g Gain/ g Protein	% of casein value	Plasma protein	Caloric intake	Caloric efficiency
	%	% calories	g	g			g/100 ml	kcal	g gain/100 kcal
Dry diets, protein efficiency ratio									
Casein, ANRC	10	9.6	76 ± 18 [1]	25.8	2.89 ± 0.46		5.4 ± 0.4		
Formula A, lyophilized	10	9.6	79 ± 19	28.3	2.79 ± 0.22	97	5.2 ± 0.3		
Formula B, lyophilized	10	9.6	59 ± 12	25.2	2.32 ± 0.30	80	5.0 ± 0.4		
Formula C, lyophilized	10	9.6	44 ± 11	22.4	1.92 ± 0.32	66	4.9 ± 0.3		
Formula D, lyophilized	10	8.9	34 ± 10	18.6	1.80 ± 0.33	62	4.3 ± 0.4		
Liquid diets, protein efficiency ratio									
Formula A	2.9	9.6	78 ± 16	27.4	2.84 ± 0.19	98	4.5 ± 0.3		
Formula B	2.9	9.6	66 ± 9	26.8	2.47 ± 0.17	85	4.3 ± 0.3		
Formula C	2.9	9.6	44 ± 12	21.6	2.01 ± 0.27	70	4.1 ± 0.6		
Formula D	2.8	8.9	29 ± 10	18.0	1.59 ± 0.28	55	4.5 ± 0.6		
Concentrated liquid diets (undiluted), caloric efficiency									
Formula A	5.0	14.9	186 ± 24				5.5 ± 0.2	1689	110 ± 4
Formula B	6.5	19.4	183 ± 28				5.4 ± 0.2	1794	102 ± 4
Formula C	6.0	17.9	170 ± 23				5.1 ± 0.1	1772	96 ± 7
Formula D	4.1	12.2	55 ± 21				4.7 ± 0.4	948	56 ± 17

[1] Values given with standard deviations.

gain/1000 kcal) than with formula B (102 g) or C (96 g) and was very low with formula D (56 g). Caloric efficiency values reflected protein quality although weight gains per se did not.

Plasma protein values, which are summarized in table 2, were lower in the groups receiving the soybean formulas of lower protein quality. When the formulas were fed as the sole article of diet and as the 10% protein dry diets, the differences in plasma protein levels between groups receiving formula A and those receiving formulas C and D were statistically significant ($P < 0.01$). When the formulas were fed as the liquid 10% protein diets, all plasma protein levels were slightly low; the reason for this is not apparent. Hemoglobin levels were similar with all diets, apparently unaffected by protein quality; these values are not shown.

The percentage of protein in the carcass was similar with all 3 types of feeding regimens and was not affected by protein quality, as shown in table 3. Carcass lipid levels were higher with the soybean formula diets than with the casein diet. With the soybean diets, carcass lipids appeared to be related to protein quality and weight gain; relatively high values with formula A and relatively low ones with formula D.

Carcass ash levels were statistically higher in the group fed formula D as compared with those fed formula A in all 3 types of diets. This difference is apparently related to the small weight gain of groups fed formula D.

The weights of the liver, kidneys, heart and adrenals were measured in all animals at killing. These were generally within normal limits when expressed as a percentage of the body weight. The weights of the thyroid glands were determined for the animls fed the concentrated soybean formulas (undiluted) as the sole article of the diet. The weights of the thyroid glands were 17, 17, 16 and 11 mg in the animals fed soybean formulas A, B, C and D, respectively. When expressed on a body weight basis the weights of the thyroid glands were 7.1, 7.2, 7.3 and 10.4 mg/100 g body weight with soybean formulas A, B, C and D, respectively. The value with formula D was significantly higher than that for the other soybean formulas. Liver lipid levels of the animals fed the dry 10% protein diets (table 3) were apparently related to protein quality. Total lipids were lowest with the casein diet (5.3%) providing high quality protein and were highest in the group receiving formula D (9.6%)

TABLE 3

Carcass composition and liver lipid levels in male weanling rats fed various soybean formulations for 4 weeks

	Protein level		Carcass composition				Liver lipid
			Protein	Lipid	Ash	Total solids	
	%	% calories	%	%	%	%	%
			Dry diets, protein efficiency ratio				
Casein, ANRC	10	9.6	19.6±1.4 [1]	12.0±2.5	3.8±0.4	34.1±1.8	5.3±0.4
Soybean formula A	10	9.6	19.1±1.3	14.5±4.6	3.8±0.4	35.7±2.4	6.3±0.7
Soybean formula B	10	9.6	19.0±1.2	13.6±2.0	4.1±0.3	35.6±1.5	7.4±1.2
Soybean formula C	10	9.6	19.1±1.0	12.6±3.6	4.3±0.4	34.7±1.5	6.7±0.6
Soybean formula D	10	8.9	18.8±1.9	11.6±2.7	4.4±0.3	33.9±1.9	9.6±2.5
			Liquid diets, protein efficiency ratio				
Soybean formula A	2.9	9.6	19.4±1.6	16.8±3.6	3.2±0.3	38.6±3.5	10.2±4.4
Soybean formula B	2.9	9.6	19.0±1.4	12.7±2.1	3.6±0.4	35.6±2.2	9.9±3.1
Soybean formula C	2.9	9.6	19.2±1.6	12.9±3.6	4.3±0.4	35.4±2.7	11.8±3.2
Soybean formula D	2.8	8.9	19.6±1.7	13.8±4.6	3.8±0.5	35.6±3.2	10.9±3.5
			Concentrated liquid diets (undiluted), caloric efficiency				
Soybean formula A	5.1	15.2	19.5±1.2	18.7±5.4	3.0±0.4	39.6±3.0	8.8±1.6
Soybean formula B	5.8	17.3	19.6±1.0	15.5±2.9	3.0±0.4	37.8±1.8	8.9±1.8
Soybean formula C	6.1	18.3	18.8±1.5	17.5±5.4	3.3±0.5	38.5±3.2	8.7±1.5
Soybean formula D	4.0	12.3	19.4±1.1	12.2±3.1	3.8±0.4	34.8±2.9	9.4±3.0

[1] Values given with standard deviations.

TABLE 4
Amino acid composition of soybean formulas

Amino acids	Formula				Formula			
	A	B	C	D	A	B	C	D
	g/100 g protein				*g/liter (1.34 kcal/ml)*			
Sulfur-containing:								
Methionine [1]	2.0	1.1	1.2	1.2	1.00	0.70	0.74	0.48
Cystine [2]	0.9	1.0	0.9	0.8	0.44	0.64	0.54	0.32
Methionine + cystine	2.9	2.1	2.1	2.0	1.44	1.34	1.28	0.80
Other essential:								
Isoleucine	4.8	4.8	4.7	4.6				
Leucine	8.3	8.1	8.2	7.9				
Lysine	6.9	6.2	6.0	6.4				
Phenylalanine	5.4	5.3	5.2	5.2	17.6	21.6	21.4	14.2
Threonine	3.7	3.5	4.2	4.1				
Tryptophan	1.0	1.1	1.2	1.2				
Valine	4.9	5.0	5.1	4.9				
Total nonessential	62.0	63.7	63.1	63.9	31.0	40.8	39.0	26.2

[1] An essential amino acid.
[2] A nonessential amino acid.

with the poorest protein quality. However, with all the liquid diets, liver lipid levels were high and showed no relationship to protein quality. The reason for this is not clear. None of the liquid diets were supplemented with vitamins and minerals as were the dry 10% protein diets. Label claims suggest that the choline level of these diets may have been marginal for the rat.

The amino acid compositions of the protein in the 4 soybean formulas are similar with the exception of the methionine levels (table 4). The protein in formula A, the only formula with added methionine, has 2.9% total sulfur amino acids (methionine and cystine) compared with totals of 2.1, 2.1 and 2.0 for formulas B, C and D, respectively. When fed as the sole article of the diet, formula A provides 1.44 g of sulfur-containing amino acids/liter of formula, whereas formulas B, C and D provide 1.34, 1.28 and 0.80, respectively.

DISCUSSION

The present results demonstrate that animals fed 10% protein liquid diets attain weight gains and protein efficiency values nearly identical to those of animals fed dry diets, although plasma protein and liver lipid levels differ from those found with the dry diets. The feeding of liquid diets circumvents lyophilization and eliminates the possibility of nutrient loss during drying. However, feeding liquid formulas requires considerably more time and effort than required with dry diets. Minimal precautions for the feeding of liquids include sterilization of the glass drinking tubes and daily feeding to minimize formula souring. Some of the formula products tended to settle rather rapidly, particularly after dilution with carbohydrate. This necessitated giving each animal a volume of formula just in excess of his needs based on the intake of formula on the previous day.

Despite the increasing and successful use of soybean formulas in the prophylaxis of allergic diseases in infants and children (4), only a few reports have appeared on the value of the protein in soybean formulas. Studies measuring the protein quality of several commercial soybean formulas,[4] using PER in rats, have been reported (5, 6). The PER reported here for both dry (lyophilized) and liquid diets are similar to values published in those reports. The present results indicate again that there is a wide variation in the protein quality of different soybean formulas.

The sulfur amino acids are the growth-limiting amino acids in soybean protein (7, 8) and the addition of methionine markedly improves soybean protein quality as measured in rats. The high PER attained with formula A apparently resulted

[4] Sobee, Mull-Soy and Soyalac.

from the addition of methionine to this formula product.

Soybean protein quality is markedly affected both by under- and over-heating (1, 9, 10). The rather wide variations in PER among formulas B, C and D may be related in part to the amount of heat used in processing. The similarity in amino acid composition does not necessarily indicate similarity in protein quality. Rios Iriarte and Barnes (10) also observed that the amino acid composition of overheated soybean protein may not reveal which amino acid(s) of soybean protein had become unavailable during processing.

In studies of the effect of dietary water, Keane et al. (11) observed that the addition of 80% water to a purified casein diet containing 9 or 12% protein significantly decreased PER, whereas the addition of 20% water increased PER over that attained with a diet containing no water. The explanation for the effects of moisture on casein protein quality remains unresolved. However, the removal of water by lyophilization from a formula product containing approximately 75% water had no apparent effect on the PER of soybean protein.

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Effectiveness of Vitamin E in Reversing Sterility of Male Chickens Fed a Diet High in Linoleic Acid [1]

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ABSTRACT To determine whether sterility of adult male chickens fed a diet high in linoleic acid and low in vitamin E was permanent or could be reversed by supplemental vitamin E, 3 groups of 8 White Leghorn males were fed for a 40-week experimental period as follows: group 1, a high linoleic acid (7.3%)-high vitamin E (166.3 mg/kg) diet; group 2, a high linoleic acid-low vitamin E (4.3 mg/kg) diet; and group 3, treated as group 2 from zero to 28 weeks and as group 1 from 28 to 40 weeks. The following results were obtained: 1) The adverse effect of the high linoleic acid-low vitamin E diet on fertility and semen concentration was confirmed. 2) The addition of vitamin E to the high linoleic acid diet at 28 weeks restored fertility to the level obtained from males receiving vitamin E throughout the trial. 3) Semen concentration was improved following the addition of vitamin E to the diet at 28 weeks. 4) No meaningful differences on semen volume, hatchability of fertile eggs, feed consumption, mortality, or body and testes weights were observed with males fed the various diets. The results indicate that the low fertility and semen concentration of male chickens fed a diet high in linoleic acid but low in vitamin E are reversible following vitamin E supplementation.

Arscott et al. (1) reported that adult White Leghorn male chickens fed a diet high in linoleic acid but low in vitamin E and ethoxyquin for a 25-week experimental period experienced low fertility and reduced semen concentration. When such diets were supplemented with vitamin E or ethoxyquin, no adverse effects were noted. Also a diet low in both linoleic acid and vitamin E did not appear detrimental. Increasing the number of sperm inseminated from males receiving a diet high in linoleic acid and low in vitamin E failed to improve fertility. Information on the permanence of sterility was not obtained.

Adamstone and Card (2) reported sterility, as evidenced by testicular degeneration, in male chickens after having been fed a vitamin E-deficient diet for a 2-year period. Although evidence for permanent sterility was not presented, the irreversible nature of sterility resulting from vitamin E-deficient diets for male chickens appears in the literature (3). In this respect, Evans and Burr (4) and Mason (5) have reported on the irreversibility of the sterility observed in male rats fed diets deficient in vitamin E; however, Bryan and Mason (6) were unable to demonstrate testicular degeneration in male mice fed diets deficient in vitamin E for 400 days. The permanence of the testicular degeneration noted with guinea pigs fed vitamin E-deficient diets (7) apparently is not known (8).

It was the purpose of the present experiment to determine whether supplemental vitamin E would reverse the sterility noted in male chickens fed a diet high in linoleic acid.

EXPERIMENTAL

Twenty-four dubbed White Leghorn cockerels hatched in April were housed in individual wire-floor cages in November, 1964. The present experiment began in March, 1965, at which time the males were divided into 2 groups of 8 and 16 each, with differences between groups minimized for body weights as well as semen volume and fertility (based on three preliminary ejaculations and inseminations). The group of 8 males was fed diet 3 (1),[2] which contained 7.3% of lino-

Received for publication August 12, 1966.

[1] Technical Paper no. 2178, Oregon Agricultural Experiment Station. Supported in part by a grant-in-aid from the American Poultry and Hatchery Federation. Presented in part at the 55th annual meeting of the Poultry Science Association, Logan, Utah, 1966.

[2] Both diets 2 and 3 were formulated to contain glucose monohydrate (Cerelose 2001, Corn Products Company, New York) and isolated soybean protein (Assay protein C-1, Skidmore Enterprises, Cincinnati) as major sources of energy and protein, respectively.

leic acid [3] and 166.3 mg of vitamin E [4]/kg of diet throughout the 40-week experimental period. The group of 16 males was fed diet 2 (1),[5] which contained 7.3% of linoleic acid and only 4.3 mg of vitamin E/kg of diet for 28 weeks, when they were divided into two equal groups with one group receiving diet 3, containing an adequate amount of vitamin E, and the other group continued for the remainder of the experiment with diet 2, deficient in vitamin E.

The preparation of the diets and the air-oxidized safflower oil, and the collection of the data, as well as the care and management of these chickens, have been described previously (1). A total of 2,651 eggs was incubated during this experiment. All males were killed in January, 1966, at which time weights of their testes were obtained.

Standard errors of the means were computed for all data, except feed consumption, with significance determined by Student's *t* test.

RESULTS AND DISCUSSION

The results of this experiment are presented in figure 1. Commencing with the twelfth week, a significant decrease in fertilizing capacity was observed for males fed the diet high in linoleic acid and low in vitamin E (fig. 1A). At 28 weeks one-half of the males in this group was fed the high linoleic acid diet adequate in vitamin E. Subsequent measurement of fertilizing capacity at 32, 36, and 40 weeks showed fertility for this group of males restored to a level comparable with the males maintained with the high vitamin E diet throughout. Fertility for males not receiving added vitamin E remained depressed but did not decrease further during this period.

No significant differences in semen volume were evident during the course of the experiment (fig. 1B). Although some difference in volume is suggested after 12 weeks, this difference is not apparent after 32 weeks. Semen concentration was adversely affected after the twelfth week for the males fed the diet high in linoleic acid but low in vitamin E (fig. 1C). Following supplementation of this diet with vitamin E, an increase in semen concentration was noted.

No meaningful differences on hatchability of fertile eggs (fig. 1D), body weight (fig. 1E) and feed consumption (fig. 1F) were noted for males on the 3 treatments. Three males died during the course of the experiment: one fed the high vitamin E diet, after 4 weeks, and two fed the low vitamin E diet, after 21 weeks.

No significant differences in the size of testes were noted for the 3 groups of males. Average weight of both testes per male was: 17.0 ± 2.5 (SE) g for cockerels fed the vitamin E-adequate diet throughout; 14.6 ± 2.2 g for the vitamin E-adequate group after 28 weeks and 15.6 ± 1.6 g for the vitamin E-deficient group.

These data, describing the adverse effects on fertilizing capacity and semen concentration of males fed a diet high in linoleic acid and low in vitamin E, are in accord with those in our previous report (1). Of particular interest is the observation that the resulting sterility is reversible following vitamin E fortification of the high linoleic acid diet. Four males that failed to fertilize any eggs during the twenty-first and twenty-seventh weeks regained their fertility following the addition of an adequate level of vitamin E in the diet at 28 weeks. In the group of males continuing to receive the vitamin E-deficient diet, four males were also sterile at 27 weeks. Two of these remained sterile for the remainder of the experiment; and fertility ranged from zero to not more than 27.3% at any one time for the other 2 males.

These results do not support the view that permanent sterility occurs in male chickens deprived of vitamin E (3), nor do they agree entirely with the observations reported for rats (4,5). Furthermore, it should be noted that depressed fertility occurred only with male chickens fed a high level of linoleic acid. Previous results (1) have shown that a diet low in both linoleic acid (0.03%) [6] and vitamin E (0.9 mg/kg) did not adversely affect the males'

[3] Obtained by including 10% of air-oxidized safflower oil (from alkaline refined safflower oil, Pacific Vegetable Corporation, San Francisco) which on analysis contained 72.6% of linoleic acid (1). As used in the text linoleic acid is from this source.

[4] This represented per kilogram of diet, 162 mg of added *d*-α-tocopheryl acetate (Myvamix, provided by Distillation Products Industries, Rochester, New York).

[5] See footnote 2.

[6] Obtained by including 10% of air-oxidized coconut oil in the diet.

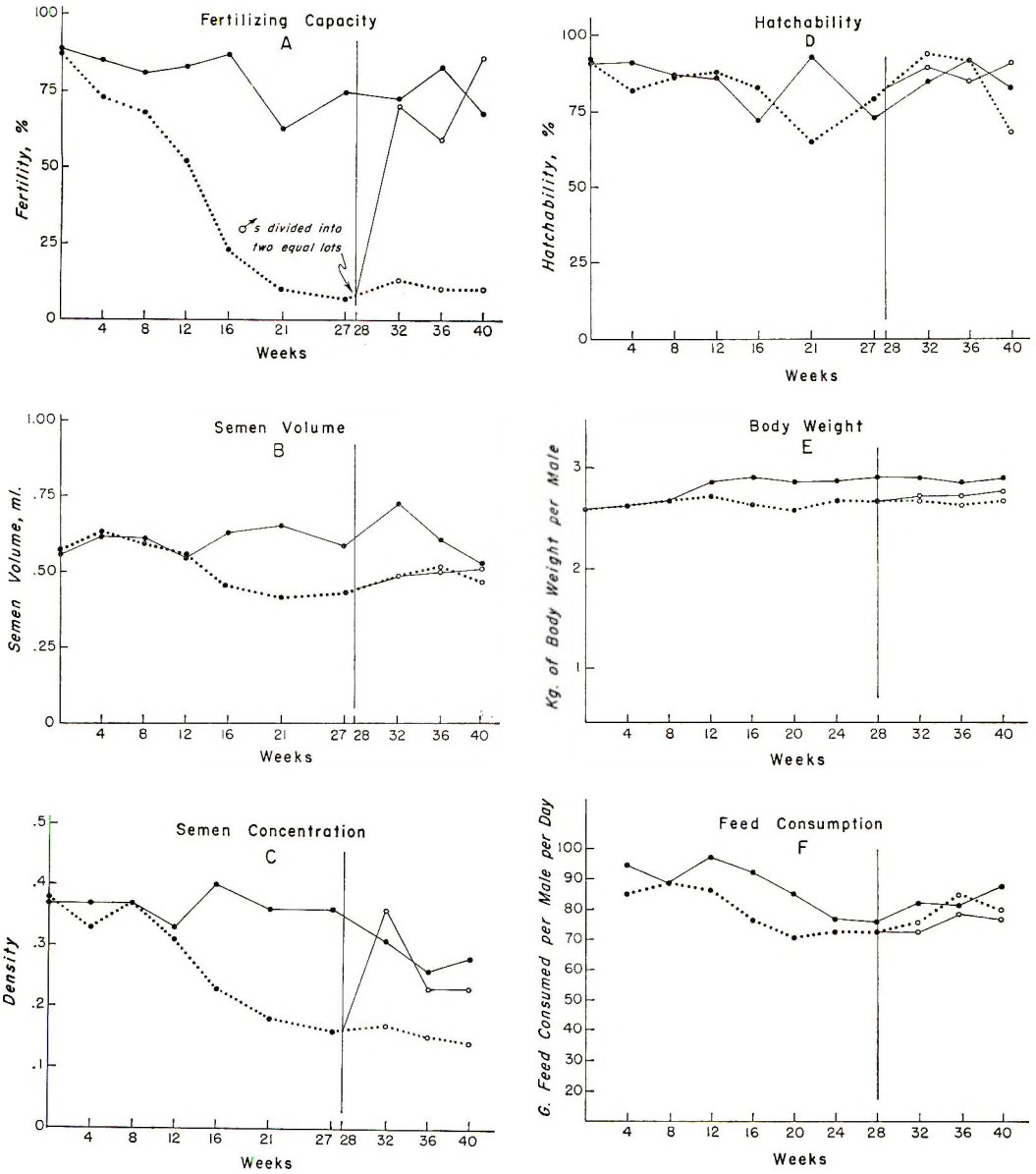

Fig. 1 Effect of linoleic acid with or without added vitamin E on (A) fertilizing capacity, (B) semen volume, (C) semen concentration, (D) hatchability, (E) body weight and (F) feed consumption. Key: ———, high linoleic acid-vitamin E diet; ·····, high linoleic acid-low vitamin E diet.

fertilizing capacity. In contrast, Mason (6) has observed that once male rats were subjected to a vitamin E-deficient diet, in which some testicular degeneration resulted, permanent sterility ensued even if these males were returned to a vitamin E-adequate diet, which points out the sensitivity of this species to a vitamin E deficiency. More recently, however, Mason (9) [7] reported that testicular degeneration in vitamin E-deficient hamsters was re-

[7] Also reported as: Mason, K. E., and S. I. Mauer 1957 Reversible testis damage in the vitamin E-deficient hamster. Anat. Rec., 127: 329 (abstract).

versed by vitamin E supplementation. As a result of this observation, he suggests that possibly more importance than necessary has been attached to the permanence of sterility in the rat due to vitamin E deficiency.

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Influence of Dietary Carbohydrates on Magnesium Utilization in the Chick [1,2]

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ABSTRACT Studies were conducted to investigate growth, mortality, feed consumption, plasma and femur magnesium levels, hemoconcentration, and magnesium balance in chicks fed diets deficient or marginally adequate in magnesium and differing in the source of carbohydrate. Four carbohydrate sources, glucose, sucrose, lactose and starch, and 4 levels of magnesium, 250, 300, 350 and 400 ppm, were fed in one or more of the studies. The results of these studies indicated that chicks fed diets containing lactose exhibited decreased mortality, increased feed and water consumption, higher plasma and femur magnesium levels, and higher hematocrit values than did chicks fed similar diets differing only in carbohydrate source. Weights of chicks fed diets containing lactose and 250 or 300 ppm of magnesium were slightly higher during the first 2 weeks of the experiment as compared with chicks fed diets containing different carbohydrate sources. Similar results were not observed for these chicks at 3 or 4 weeks of age nor for chicks fed 350 or 400 ppm of magnesium at any age, however. A final study measured magnesium balance in chicks fed either a glucose diet ad libitum, a lactose diet pair-fed to the glucose diet, or a lactose diet ad libitum. These studies suggested that one means by which lactose exerts a beneficial effect in diets marginal in magnesium content is through increased feed, and hence, increased magnesium consumption. Hemoconcentration may also be a factor partially responsible for the increased plasma magnesium levels of chicks fed the lactose diets.

Monson et al. (1), using rate of growth as a measure of the nutritional efficiencies of several carbohydrate sources in chicks, reported that lactose was utilized to a lesser extent than was dextrin, glucose, or sucrose. It is generally concluded that poultry are unable to utilize appreciable amounts of lactose as a dietary source of carbohydrate because of the bird's inability to hydrolyze this sugar in adequate amounts.

The mechanism whereby lactose and certain other sugars influence the absorption of alkaline earth cations has been the subject of extensive research (2–6). Other carbohydrates, in addition to lactose, which have been demonstrated to enhance the gastrointestinal absorption of radiocalcium and radiostrontium in the rat include cellobiose, sorbose, ribose, xylose, raffinose, melibiose, glucosamine, manitol, and sorbitol (2). Glucose, sucrose, fructose, and galactose have also been shown to stimulate the absorption of ^{45}Ca from ligated ileal segments in the rat (5).

Lengemann (7) reported that lactose could stimulate calcium absorption only if both were present in the same segment of the ileum. A similar effect of lactose was observed on magnesium, strontium, barium, and radium absorption. It appears that many carbohydrate sources may be able to stimulate the absorption of alkaline earth cations from the ileum provided they have had sufficient time to exert their influence prior to being absorbed.

The research presented in this report was designed to study the effects of several carbohydrate sources on the utilization of magnesium in chick diets deficient or marginal in magnesium content.

EXPERIMENTAL

One-day-old White Rock cockerels were used in each of the first 3 studies reported; Hubbard White Mountain cockerels were used in the final study. All chicks were wingbanded and placed in electrically heated housing units with raised wire floors. Feed and deionized water were provided ad libitum unless otherwise indicated. In the growth studies, all birds were weighed individually at weekly intervals.

Received for publication August 3, 1966.
[1] Journal Paper no. 2881, Purdue University Agricultural Experiment Station.
[2] This work was supported in part by funds from Basic, Inc., Cleveland.

TABLE 1

Basal diet composition [1]

	%
Isolated soybean protein	30.00
Soybean oil	5.00
Cellulose	3.00
Glucose monohydrate	3.87
DL-Methionine	0.50
Glycine	0.30
Choline chloride (70%)	0.20
Butylated hydroxytoluene (25%)	0.05
Mineral mix [2]	6.08
Vitamin mix [3]	1.00

[1] The ingredients listed account for 50% of the final diet formulation, and the remaining 50% was provided by the carbohydrate being studied.

[2] The mineral premix provided the following reagent minerals in g/kg of diet: $CaCO_3$, 19.10; $Ca(H_2PO_4)_2 \cdot H_2O$, 21.15; K_2HPO_4, 11.20; NaCl, 8.4; $FeSO_4$, 0.2; $ZnCO_3$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.015; $MnSO_4 \cdot H_2O$, 0.51; KI, 0.04; $Na_2MoO_4 \cdot 2H_2O$, 0.0025.

[3] The vitamin premix provided the following units of vitamins per kg of diet: (in milligrams) riboflavin, 9; thiamine·HCl, 6; Ca D-pantothenate, 20; niacin, 50; pyridoxine·HCl, 8; folic acid, 2; biotin, 0.3; menadione sodium bisulfite, 2; inositol, 1000; and vitamin B_{12}, 20 μg; and vitamin A, 25,000 USP units; vitamin D_3, 1200 ICU; vitamin E, 17.6 IU.

The composition of the basal diet is presented in table 1. This accounts for 50% of the final diet, the remaining 50% being either glucose monohydrate, sucrose, or starch. When lactose was included, it was added to the diet at a level of 25%, together with 25% glucose. Although this series of diets contains some glucose, they will be designated simply as lactose diets. Dietary magnesium levels were varied by supplementation with magnesium carbonate added at the expense of glucose.

All magnesium determinations reported herein were made with the aid of an atomic absorption spectrophotometer.[3] Standard solutions were prepared in the same manner as the test solutions. The magnesium content of the various diets was determined by wet-ashing approximately 2 g of feed with 10 ml of concentrated HNO_3, followed by dilution with deionized water to provide a suitable working range for analysis. Blood samples were collected by cardiac puncture, using heparin as an anticoagullant. Plasma was separated by centrifugation and the magnesium content determined by the method of Willis (8). Bone ash and bone magnesium analyses were determined from dry, fat-free femurs. Atomic absorption spectrophotometric determination of femur magnesium was accomplished by dissolving the ash in 5 ml of 3 N HCl followed by dilution to the appropriate volume. The magnesium content of the homogenized solutions of excreta was determined by digesting an aliquot of the excreta in concentrated HNO_3 followed by dilution to the proper volume. Strontium chloride (0.1%) was added to the dissolved bone and excreta solutions to prevent phosphate interference. Hematocrit determinations were conducted on blood drawn into heparinized capillary tubes from wing veins of chicks.

Statistical procedures used for treating data obtained in these studies were conducted by analyses of variance (9). Comparisons of treatment means were made by the sequential method of Newman and Keuls (9), with the harmonic means serving as the average number of observations in those cases involving unequal subclass numbers. In trial 3 where only 2 treatment means were compared, Student's *t* test was used.

Trial 1. This study was designed to observe growth, feed consumption, mortality, and femur and plasma magnesium levels of birds maintained with rations containing different carbohydrate sources and different levels of magnesium. Four hundred and eighty chicks were assigned at random to 48 pens of 10 chicks per pen. Three replicates of 10 birds each were then assigned at random to each of the diets. A 4×4 factorial arrangement of treatments (table 2), which included 4 levels of magnesium (250, 300, 350, and 400 ppm, calculated) and 4 sources of carbohydrate (glucose, starch, sucrose, and lactose) was used in this experiment. The birds were fed their respective rations for 4 weeks, at which time the experiment was terminated and femur and plasma samples were collected for magnesium determinations.

Trial 2. This experiment was conducted in a manner similar to the previous one with the exception that only 2 sources of carbohydrate (glucose and lactose) were used. Four hundred and forty birds were assigned at random to the 8 experimental diets (table 3); 60 birds per diet for the 250- and 300-ppm magnesium diets and 50 birds per diet for the 350- and 400-ppm

[3] Model 303, Perkin Elmer Corporation, Norwalk, Connecticut.

TABLE 2

Effect of diet on growth, mortality, and femur and plasma magnesium levels at 4 weeks (trial 1)

Treatment	Carbohydrate	Magnesium	Mortality	Avg wt [1]	Feed [1,2] consumed	Magnesium levels [1,3]	
						Femur	Plasma
		ppm		*g*		*% of ash*	*mg/100 ml*
1	Glucose	250	27/30	353.3 [a]	315.3 [a]	0.47 [af]	0.65 [adh]
2	Glucose	300	21/30	480.7 [bcef]	447.0 [ab]	0.52 [abf]	1.14 [abdh]
3	Glucose	350	8/30	510.0 [bcef]	635.3 [cdeh]	0.53 [acf]	1.14 [acdh]
4	Glucose	400	5/30	510.7 [bcef]	621.3 [def]	0.54 [adf]	1.53 [bcefijk]
	Avg			463.7	504.7	0.52	1.12
5	Starch	250	23/30	315.7 [a]	301.0 [i]	0.37 [ei]	0.61 [dh]
6	Starch	300	8/30	455.3 [cef]	555.3 [bef]	0.47 [efi]	1.23 [adeh]
7	Starch	350	2/30	488.3 [bcef]	645.3 [cdeh]	0.54 [afg]	1.07 [adfh]
8	Starch	400	0/30	460.7 [cdef]	595.0 [ef]	0.66 [hjkl]	1.70 [bcefgijk]
	Avg			430.0	524.2	0.51	1.15
9	Sucrose	250	24/30	327.7 [a]	173.3 [j]	0.37 [i]	0.57 [h]
10	Sucrose	300	13/30	450.3 [ef]	495.3 [af]	0.49 [af]	1.28 [adhi]
11	Sucrose	350	3/30	539.7 [bcef]	610.7 [efg]	0.57 [afj]	1.15 [adhj]
12	Sucrose	400	4/30	572.7 [bdgh]	642.3 [cdeh]	0.60 [bcdgkl]	1.47 [bcefijk]
	Avg			472.6	480.4	0.51	1.12
13	Lactose	250	19/30	346.7 [a]	552.0 [bfk]	0.55 [afl]	1.14 [adhk]
14	Lactose	300	6/30	448.7 [f]	738.7 [cdg]	0.62 [bcdgjlm]	1.67 [bcefijkl]
15	Lactose	350	3/30	477.3 [cefg]	917.3 [k]	0.68 [hkm]	1.80 [bcefijkm]
16	Lactose	400	6/30	469.0 [cefh]	744.0 [cd]	0.73 [h]	2.21 [glm]
	Avg			435.4	738.0	0.65	1.71

[1] Means with the same lettered superscript in a vertical column are not statistically different ($P > 0.05$).
[2] Expressed in grams on a chick-day basis as average feed consumed per chick.
[3] Average of 5 samples (femur); 6 samples (plasma), where possible.

magnesium diets. Six birds from each treatment, where possible, were killed at weekly intervals for 4 consecutive weeks for femur and plasma magnesium determinations.

Trial 3. This experiment was designed to determine the extent to which the differences in plasma magnesium concentrations observed in chicks fed lactose and glucose diets were due to differences in hemoconcentration. Diets identical to the glucose and lactose diets containing 300 ppm magnesium in trial 2 were used in this experiment. One hundred and forty one-day-old chicks were used in this study, 70 per treatment. Hematocrit and plasma magnesium determinations were conducted at weekly intervals for 4 consecutive weeks.

Trial 4. A final study was conducted to measure magnesium balance in chicks fed either a glucose diet ad libitum, a lactose diet pair-fed to the glucose diet, or a lactose diet fed ad libitum. The diets used in this study contained 350 ppm magnesium. One hundred and twenty one-day-old chicks were assigned at random to 12 pens of 10 each, four pens per treatment. The balance study was initiated when the chicks were 12 days of age. The total feed consumed and excreta voided were determined for the 10 chicks in each

TABLE 3

Effect of diet on femur and plasma magnesium levels at weekly intervals (trial 2)

Treatment	Carbohydrate	Magnesium	Magnesium levels [1,2]							
			Femur				Plasma			
			Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
		ppm	*% of ash*				*mg/100 ml*			
1	Glucose	250	0.35 [a]	0.24 [a]	0.21 [a]	0.36 [a]	0.44 [ab]	0.68 [a]	0.56 [a]	0.76 [a]
2	Glucose	300	0.32 [a]	0.26 [ab]	0.26 [ab]	0.41 [a]	0.39 [b]	0.89 [ab]	0.99 [bc]	0.97 [ab]
3	Glucose	350	0.36 [a]	0.34 [bcd]	0.37 [bce]	0.58 [bc]	0.67 [abc]	1.18 [bc]	1.16 [bc]	1.46 [ac]
4	Glucose	400	0.34 [a]	0.40 [cd]	0.48 [cd]	0.63 [bc]	0.88 [c-f]	1.28 [bc]	1.25 [bc]	1.71 [bcd]
	Avg		0.34	0.31	0.33	0.36	0.60	1.01	0.99	1.23
5	Lactose	250	0.29 [a]	0.32 [ad]	0.32 [ae]	0.57 [c]	0.69 [abd]	1.21 [bc]	0.84 [ac]	1.47 [ad]
6	Lactose	300	0.37 [a]	0.37 [cd]	0.47 [cf]	0.69 [bdc]	0.53 [abe]	1.36 [bc]	1.31 [bc]	1.96 [cd]
7	Lactose	350	0.30 [a]	0.43 [c]	0.53 [dfg]	0.70 [bce]	0.68 [abf]	1.51 [c]	1.82 [d]	1.90 [cd]
8	Lactose	400	0.41 [a]	0.50 [e]	0.63 [g]	0.81 [de]	1.22 [g]	1.36 [bc]	1.86 [d]	2.07 [cd]
	Avg		0.34	0.41	0.49	0.69	0.78	1.36	1.46	1.85

[1] Average of 6 samples, where possible.
[2] Means with the same lettered superscript in a vertical column are not statistically different ($P > 0.05$).

pen for 2 periods of 48 hours' duration. The excreta were collected in flat stainless steel pans containing deionized water. The total excreta collections were homogenized in a large Waring blender, made to volume and aliquots were taken for magnesium analysis as indicated previously.

RESULTS

The effects of the various treatment combinations on chick weight, feed consumption, mortality, and femur and plasma magnesium levels at 4 weeks of age are presented in table 2. Irrespective of diet, mortality was associated with the tetany syndrome characteristic of magnesium deficiency. Mortality was extremely high for birds fed the diets containing 250 ppm magnesium, although the lowest rate was observed for chicks fed the diet containing lactose. In these studies deficiency symptoms first became apparent by the second or third day of treatment, with mortality reaching its highest peak during the fourth to sixth days of the experiment for those birds fed the glucose, starch, and sucrose diets. The inclusion of lactose in the diet appeared to delay the onset of magnesium deficiency since appreciable mortality was not observed until the second week of the experiment. Diarrhetic conditions in chicks fed lactose in their diets became apparent by the end of the first week.

Statistical analysis of the 4-week weights showed a significant effect of magnesium level ($P < 0.01$), a significant effect of carbohydrate source ($P < 0.05$), and a significant magnesium $\times$ carbohydrate interaction ($P < 0.01$). At the 350- and 400-ppm levels of magnesium, growth was most rapid in chicks fed the diets containing sucrose. Growth was intermediate in chicks fed diets containing glucose and was slowest in chicks fed diets containing starch and lactose. At the end of 4 weeks, growth had reached a plateau at the 350-ppm magnesium level in chicks fed the glucose, starch and lactose rations. With the diet containing sucrose, however, growth was still increasing at the 400-ppm level of dietary magnesium.

Statistical analyses were also conducted on the 1-, 2-, and 3-week weights. In addition to decreased mortality of chicks fed diets containing lactose at the two lower levels of magnesium, chick growth during the first and second weeks was likewise influenced favorably by lactose. Chicks fed the lactose diets containing either 250 or 300 ppm magnesium were larger than those fed similar diets differing only in source of carbohydrate. These

differences were not statistically significant in most instances, however.

Birds fed the lactose diets containing 250, 300 or 350 ppm magnesium showed significant increases ($P < 0.05$) in feed intake when compared with birds fed similar diets containing glucose, starch, or sucrose. At the highest dietary level of magnesium (400 ppm), differences in feed consumption between birds fed lactose, glucose, and sucrose were not significant ($P > 0.05$). Although no precise measure of water consumption was made in these studies, a markedly increased water intake was observed for chicks fed lactose in their diets as compared with chicks fed the other sources of carbohydrate.

The 4-week plasma and femur magnesium concentrations of birds fed the diets containing glucose, starch, and sucrose were similar at each dietary level of magnesium. Chicks fed the lactose-containing diets, however, exhibited consistantly higher femur and plasma magnesium concentrations at each level of dietary magnesium. Increased dietary levels of magnesium generally resulted in increased levels of femur and plasma magnesium.

As a result of the marked influence of lactose on femur and plasma magnesium concentrations observed in this study, a second study was conducted to observe weekly changes in the magnesium content of these tissues (table 3). The same levels of magnesium as fed in the first experiment were studied in this experiment; however, only glucose and lactose were fed as carbohydrate sources. Higher levels of plasma magnesium in chicks fed the lactose-containing diets as compared with chicks fed the glucose-containing diets became evident as early as day 7 of the experiment. Higher plasma magnesium concentrations were observed at each level of dietary magnesium in chicks fed the lactose-containing diets as compared with chicks fed the glucose-containing diets after 2, 3 or 4 weeks of the experiment. Femur magnesium levels were not greatly different after the first week of the study, but significant differences were observed after the second week and for the remainder of the experiment. The femurs of the chicks fed the lactose diets increased in magnesium concentrations (as % of bone ash) throughout the experiment. The femurs of chicks fed the glucose diets accumulated considerably less additional magnesium (as % of bone ash) throughout the experiment.

Trial 3 was designed to determine the extent to which the differences in plasma magnesium levels observed in the two previous studies were due to differences in hemoconcentration. Comparisons were made with chicks fed diets containing glucose or glucose plus 25% lactose as dietary sources of carbohydrate. Each diet contained 300 ppm magnesium, a level which has been demonstrated in the previous experiments as marginal for the chick. Hematocrit percentages were higher at each weekly interval for birds fed the lactose diet, but significant differences ($P < 0.01$) were observed only at 2 weeks (table 4). If hematocrit and plasma magnesium levels at 2 weeks are considered, where differences in hemoconcentration are shown to be significant, only 35% of the increased plasma magnesium level in the lactose-fed birds may be attributed to hemoconcentration. Plasma magnesium levels of the birds fed the lactose diet, however, were approximately 80% higher than were observed in birds fed the glucose diet. These data indicate that differences in the levels of plasma magne-

TABLE 4

Effect of diet on hematocrit and plasma magnesium levels at weekly intervals (trial 3)

Treatment	Carbohydrate	Hematocrit				Plasma Mg			
		Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
		%	%	%	%	*mg/100 ml*		*mg/100 ml*	
1	Glucose	25.2	23.9 **	28.5	26.2	0.36	0.68 **	1.01 **	1.33 **
2	Lactose	26.8	30.9 **	30.1	27.0	0.56	1.23 **	1.35 **	1.86 **

[1] Average of 8 samples (hematocrit); 6 samples (plasma), where possible.
** $P < 0.01$.

TABLE 5

Effect of diet on magnesium balance in the chick (trial 4) [1,2]

	Treatment		
	Glucose	Lactose, pair-fed	Lactose, ad libitum
Feed consumed/ chick, g	60.8 [a]	58.7 [a]	71.0 [b]
Mg consumed/ chick, mg	20.6 [a]	19.8 [a]	24.0 [b]
Mg retained/ chick, mg	12.1 [a]	10.1 [a]	13.2 [a]
Mg retained/g gain, mg	0.34 [a]	0.35 [a]	0.43 [a]
Plasma Mg, mg/100 ml	1.01 [a]	1.33 [b]	1.43 [b]
Femur Mg, % bone ash	0.50 [a]	0.62 [b]	0.69 [c]
Final chick wt, g	372.2 [a]	255.0 [b]	341.1 [a]

[1] Balance trials were initiated when chicks were 12 days of age.
[2] Means with the same lettered superscript in a horizontal row are not statistically different ($P > 0.05$).

sium observed in these studies are not entirely the result of hemoconcentration.

The results of the growth study (trial 1) indicated that chicks fed diets containing 25% lactose consumed significantly greater amounts of feed than chicks fed similar diets differing only in carbohydrate source. A final study was conducted to measure magnesium balance in chicks fed either a glucose diet ad libitum, a lactose diet pair-fed to the glucose diet, or a lactose diet fed ad libitum. In this study, the magnesium content of the diets was 350 ppm. The results of this study are presented in table 5 where values in the table represent the means of eight separate balance trials. Feed consumption, magnesium consumption and retention data are means for 48-hour periods of the balance trials.

Birds fed the lactose diet ad libitum exhibited significantly higher feed consumption ($P < 0.05$) and, as a consequence, higher magnesium consumption than did either the groups of chicks fed the glucose diet ad libitum or the lactose diet pair-fed. The magnesium retention, when expressed as milligrams of magnesium retained per gram of weight gain, was similar for chicks fed the glucose diet or the lactose diet pair-fed to the glucose diet. Chicks fed the lactose diet ad libitum showed higher magnesium retention, but this was not shown to be statistically significant due to considerable variation between trials.

Plasma and femur magnesium determinations made at the conclusion of the experiment again showed higher blood and bone levels of this element for chicks fed the lactose diet, regardless of whether the diet was fed ad libitum or pair-fed to the amount consumed by the chicks receiving the glucose diet ad libitum. The chicks pair-fed the lactose diet were considerably smaller, however, than chicks fed the lactose or glucose diets ad libitum. Similar plasma and femur magnesium levels are observed in chicks fed the glucose diet and the lactose diet pair-fed if a correction is made for the smaller size of the pair-fed birds (body weight/100). Correction for differences in body weight and total feed consumption for birds fed the lactose and glucose diets ad libitum, however, still show higher plasma and femur magnesium in birds fed the lactose diet ad libitum.

DISCUSSION

The changes in blood and bone magnesium during periods of magnesium deficiency observed in this study with chickens are in agreement with previous work conducted with other species (10–14). Results of trial 2 show a sharp decline in plasma magnesium during the first week of magnesium deficiency. Including lactose in a chick's diet considered deficient or marginally adequate in magnesium results in a less severe drop in plasma and femur concentrations, when compared with similar diets containing glucose, starch, or sucrose. Possible mechanisms by which lactose exerts these effects on the utilization of alkaline earth elements have been suggested by several groups of workers. The enhancing action of lactose on calcium absorption has been demonstrated clearly in the rat. Bergeim (15) suggests the effect of lactose on calcium absorption as that of promoting an increased acidity of the intestinal contents. Other work, however, has demonstrated that lactose reduced the intestinal pH in rats fed a vegetable diet but not in rats

fed a meat diet where calcium retention was also observed to be improved (16). Other mechanisms such as the combination of lactose with Ca^{++} or Mg^{++} ions to form soluble, nonionized compounds, (17), and the formation of a lactose-calcium complex (3) have been proposed. The more recent work of Wasserman and Comar (2) and Wasserman and Lengemann (6), however, suggests that the effect of lactose on alkaline earth absorption is mediated through an increased permeability of the intestinal membrane or through an unknown intermediate.

Although lactose is not considered as a carbohydrate which birds would be expected to rely on naturally, these studies indicated that this sugar exerts a beneficial effect on magnesium utilization in the chick fed marginal levels of magnesium. A favorable effect is shown by decreased mortality during the first weeks of a magnesium deficiency. The addition of 25% lactose to diets containing 250, 300, 350, and 400 ppm magnesium resulted in slight increases in chick growth during the first 2 weeks of life as compared with chicks fed diets containing similar levels of magnesium but differing only in the source of carbohydrate. It was also shown that during periods of magnesium deficiency, lactose exerts a favorable influence on maintaining plasma and femur magnesium concentrations at significantly higher levels than do similar diets containing glucose, starch, and sucrose. Hemoconcentration as a major factor accounts for only part of these differences. It is emphasized that with diets adequate in magnesium, lactose will not sustain growth at a rate comparable to that of chicks fed a more readily metabolizable source of carbohydrate.

The present studies indicate that the enhancing effect of lactose in circumventing a more severe magnesium deficiency in the chick may be directly related to increased feed intake. Since lactose is poorly utilized for energy by the chick, more feed is required to obtain similar amounts of energy as compared with chicks fed the non-lactose diets. In terms of absolute amounts of magnesium, birds fed lactose have much more magesium at their disposal for absorptive purposes. These studies do not disregard possible effects of lactose on promoting increased intestinal absorption of magnesium. The results of the balance study indicated that when the lactose diet was fed ad libitum, correction for body weight and feed consumption did not account for the increased plasma and femur magnesium levels observed in these birds.

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Influence of Lactose and Glucose on Magnesium-28 Retention in the Chick [1]

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ABSTRACT The utilization of orally or intraperitoneally administered ^{28}Mg was studied in chicks fed diets containing 300 ppm magnesium and either glucose or lactose (25% of diet) plus glucose as carbohydrate sources. No significant influence of the dietary carbohydrate was observed on the retention of orally or intraperitoneally administered ^{28}Mg in the whole chick. Radioactivity in the blood at the initial withdrawal and in the femur at the termination of the experiments, however, indicated an increased concentration of ^{28}Mg in these tissues for chicks fed lactose in their diets. These differences were statistically significant only for chicks receiving the isotope by oral administration. The radioactivity in the blood and femur of chicks given intraperitoneal injections of ^{28}Mg was not significantly altered by the source of carbohydrate, although these values were higher for birds fed lactose in their diets. Lactose, in addition to its influence on increased feed consumption and hemoconcentration which were previously reported, appears also to exert a stimulatory influence on magnesium absorption in the chick.

Previous studies in this laboratory have shown that the inclusion of 25% lactose in a chick's diet which is deficient or marginally adequate in magnesium resulted in decreased mortality, increased feed consumption, and higher plasma and femur magnesium levels than observed for chicks fed similar diets differing only in carbohydrate source (1). It was suggested that increased consumption of the lactose diets was a major factor contributing to the decreased mortality and elevated blood and bone concentrations of this mineral element.

Although extensively studied, the mechanism whereby lactose and possibly other carbohydrate sources affects the absorption of alkaline earth cations is not known precisely. More recent reports suggest that the "lactose effect" on alkaline earth absorption is mediated through increased permeability of the intestinal membrane or through an unknown intermediate (2, 3). Other work with rats has led to the suggestion that magnesium may be absorbed via the same mechanism (s) as those studied with calcium, strontium, barium, and radium (4, 5). This has not been demonstrated to occur in the chick, although there is evidence which shows that lactose did not increase the gastrointestinal absorption of ^{45}Ca in the chick as it did in the rat (6).

The present study was designed to investigate the influence of dietary lactose and glucose on ^{28}Mg utilization by the chick. The objectives of the two short-term isotope studies were to determine the retention of oral and intraperitoneal administered doses of ^{28}Mg, and to discern the relative concentrations of this isotope in the blood at specified experimental intervals and in the bone at the termination of the experiments.

EXPERIMENTAL

Two sources of carbohydrate, glucose and lactose (25% of diet) plus glucose, were added to a basal ration identical to that used in previous studies concerned with magnesium utilization in the chick (1). Both diets were supplemented with magnesium carbonate to provide a total dietary level of 300 ppm of magnesium.

Trial 1. Two groups of one-day-old White Leghorn cockerels were wing-banded, weighed, and placed in wire-floor, electrically heated chick batteries. Feed and deionized water were provided ad libi-

Received for publication August 3, 1966.
[1] Journal paper no. 2882, Purdue Agricultural Experiment Station, Lafayette, Indiana.

tum. On the eighth day, 40 chicks (20 chicks/treatment) of uniform weight were transferred to individual stainless steel metabolism cages. On the fourteenth day, 24 chicks (12 chicks/treatment) were orally dosed with 3 μCi of ^{28}Mg [2] in 0.5 ml of dilute HCl in saline. Each dose contained approximately 160 μg of stable magnesium. Average weights of the chicks fed the glucose and lactose diets were 101.3 and 101.5 g, respectively. Immediately following the administration of the isotope, and at intervals of 4, 8, 12, 16, 24, and 48 hours after ^{28}Mg administration, total body radioactivity was determined in a whole-animal, gamma-scintillation counter.[3] One milliliter of blood was withdrawn by means of cardiac puncture into heparinized needles and syringes at intervals of 8, 16, 24, and 48 hours. ^{28}Mg activity of the blood was determined in a deep-well scintillation detector [4] equipped with a sodium iodide crystal.

Plasma magnesium levels were determined from birds maintained with the same diets but not dosed with ^{28}Mg. Blood was collected by cardiac puncture using heparinized needles and syringes, the plasma separated by centrifugation, and the magnesium content determined by atomic absorption spectrophotometry as described by Willis (7).

Studies were also conducted to determine the retention of ^{28}Mg which had been administered intraperitoneally. Sixteen birds (8 birds/treatment) were injected intraperitoneally with 3 μCi of ^{28}Mg in 0.5 ml of dilute HCl in saline. Average weights of the chicks fed the glucose and lactose diets were 102.3 and 100.9 g, respectively. Measurements of the radioactivity in the chick, but not in blood, were determined as described previously.

The left femur was removed from all chicks at the termination of the experiment (48 hours). The femurs were freed of adhering tissue, weighed, and ashed in a muffle furnace for 16 hours at 600°. Each ashed bone was dissolved in 2 ml of 3 N HCl. The dissolved bone plus 20 ml of XDC scintillation fluid were placed in counting vials and measured for radioactivity in a liquid scintillation detector.[5]

Trial 2. Two diets identical to those in trial 1 were fed in this experiment. This study differed, however, from the previous one in 3 respects: 1) Hubbard White Mountain cockerels were used as experimental animals; 2) chicks were not maintained in individual cages; and 3) the experiment was terminated at 4 hours. At 12 days of age, 20 birds fed the glucose diet (average weight 126 g) and 20 birds fed the lactose diet (average weight 115 g) were selected for the isotope study.

Ten birds fed the glucose diet and 10 birds fed the lactose diet were orally dosed with 1.2 μCi of ^{28}Mg [6] in 0.5 ml of dilute HCl in saline. Each dose contained approximately 190 μg of stable magnesium. Total body ^{28}Mg activity was measured in each chick immediately following dosing and 4 hours post-dosing as described in trial 1. After counting the radioactivity in the whole chick at 4 hours, samples of blood were collected for hematocrit (obtained by wing vein puncture) and whole-blood ^{28}Mg determinations (obtained by cardiac puncture). The left femur was removed from each bird at this time and prepared for ^{28}Mg determinations as described previously.

Ten birds fed the glucose diet and 10 birds fed the lactose diet were also injected intraperitoneally with 1.2 μCi of ^{28}Mg in 0.5 ml of dilute HCl in saline. Radioactivity in the body immediately following injection and in the body, blood and femur at 4 hours was measured as previously described.

Statistical analyses were conducted using Student's *t* test as the test criterion (8).

RESULTS

Data of radioactivity in the whole body, blood and femur, and plasma magnesium levels of chicks in trial 1 are presented in table 1. The source of carbohydrate fed did not significantly alter ($P > 0.05$) the retention of either oral or intraperitoneal doses of ^{28}Mg in the whole chick at any of the intervals following ^{28}Mg administration. Greater proportions of the administered doses of ^{28}Mg were retained at all

[2] Brookhaven National Laboratory, Upton, L. I., New York.
[3] Armac scintillation detector, model 440, Packard Instrument Company, Inc., Downers Grove, Illinois.
[4] Model 442, Packard Instrument Company.
[5] Model 314 EX (Tri-Carb), Packard Instrument Company.
[6] See footnote 2.

TABLE 1

Radioactivity in the whole body, blood, and femur; and plasma magnesium levels in chicks fed diets containing either glucose or lactose (25%) + glucose (trial 1)

	Hours after ^{28}Mg administration						
	2	4	8	12	16	24	48
Whole body radioactivity [1]							
Lactose, oral ^{28}Mg	—	62.8	58.4	56.1	55.7	53.4	48.7
Glucose, oral ^{28}Mg	—	64.7	57.4	54.5	54.1	53.7	49.3
Lactose, injected ^{28}Mg	90.0	89.6	87.9	87.9	84.4	80.9	75.9
Glucose, injected ^{28}Mg	91.8	92.0	89.7	85.2	85.8	84.1	77.9
Whole blood radioactivity [2]							
Lactose, oral ^{28}Mg	—	—	1443 *	—	1079	1105	1388
Glucose, oral ^{28}Mg	—	—	984	—	1104	1238	1356
Femur radioactivity							
Lactose, oral ^{28}Mg	—	—	—	—	—	—	5034 *
Glucose, oral ^{28}Mg	—	—	—	—	—	—	4024
Lactose, injected ^{28}Mg	—	—	—	—	—	—	7151
Glucose, injected ^{28}Mg	—	—	—	—	—	—	6589
Plasma Mg, mg/100 ml							
Lactose	—	—	—	—	—	—	1.61 **
Glucose	—	—	—	—	—	—	0.66

[1] Expressed as % of the original dose retained after correction for background and decay.
[2] Expressed as counts/min/ml whole blood; background and decay corrected.
[3] Expressed as counts/min/g bone; background and decay corrected.
*, ** Significantly different from chicks fed the glucose-containing diet ($P < 0.05$ and $P < 0.01$, respectively).

time intervals in chicks given the isotope by intraperitoneal injection as compared with chicks given the isotope orally.

Although carbohydrate source did not appear to significantly alter magnesium absorption or retention, or both, when measured as a percentage of the orally administered dose retained in the whole chick, birds fed the diet containing 25% lactose exhibited significantly higher ^{28}Mg activity in the blood ($P < 0.05$) at the end of 8 hours when compared with that in birds fed only glucose as the dietary source of carbohydrate. Differences in the ^{28}Mg activity of the blood from the 2 groups of chicks were not statistically significant at 16, 24, and 48 hours post-administration ($P > 0.05$).

The higher ^{28}Mg activity in the femurs of chicks fed the lactose diet indicated further that this carbohydrate may act in some manner to alter the utilization of this element in the chick. When the isotope was administered orally, significantly greater amounts of radioactivity ($P < 0.05$) were found after 48 hours in the femurs of chicks fed lactose as compared with those fed the diet containing only glucose. A similar trend was shown when the isotope was administered intraperitoneally but the differences observed were not statistically significant ($P > 0.05$). Significantly higher plasma magnesium levels were observed in chicks fed the lactose diet as compared with chicks fed the glucose diet (1.61 vs. 0.66 mg/100 ml).

Since the only significant difference observed in blood radioactivity between chicks fed the 2 diets in trial 1 was at the earliest time interval measured (8 hours), it was thought that a dietary influence may have been manifesting itself at some earlier time. A second study was initiated to determine the ^{28}Mg activity in the whole body, blood, and femurs of chicks fed the glucose and lactose diets 4 hours after administration of the isotope (table 2).

As observed in trial 1, the source of carbohydrate fed did not greatly alter the retention in the whole chick of either orally or intraperitoneally administered doses of ^{28}Mg at 4 hours post-administration. The retention of orally administered ^{28}Mg in the whole chick in trial 2 was considerably higher in both groups as compared with that observed in trial 1. In trial 1, chicks were offered feed ad libitum prior to and following ^{28}Mg dosage. In

TABLE 2

^{28}Mg activity in the chick, blood, and femur at 4 hours after ^{28}Mg administration, and hematocrit levels (trial 2)

	Diet	
	Lactose	Glucose
Total body radioactivity [1]		
Oral ^{28}Mg, % dose	81.0	82.9
Injected ^{28}Mg, % dose	89.0	90.1
Blood radioactivity [1]		
Oral ^{28}Mg, % dose/ml $\times 10^3$	6.6**	5.3
Injected ^{28}Mg, % dose/ml $\times 10^3$	7.6	7.0
Femur radioactivity [1]		
Oral ^{28}Mg, cpm/g	3418**	2363
Injected ^{28}Mg, cpm/g	4457	4107
Hematocrit, %	31.8**	25.7

[1] All counts are corrected for decay and background.
** Significantly different from chicks fed the glucose-containing diet ($P < 0.01$).

trial 2, in an attempt to more closely regulate the amount of food in each chick's gastrointestinal tract and thereby possibly minimize individual chick variation, the chicks were fasted for 4 hours and then one hour before isotope administration were again offered feed ad libitum. Feed was also offered ad libitum after isotope administration. It is not clear whether these differences in feeding of the chicks in trials 1 and 2 account for the observed differences in whole-body retention of ^{28}Mg and the extent to which feed in the gastrointestinal tract influences the passage and absorption of the ^{28}Mg.

The radioactivity in the blood and femur 4 hours post-administration was significantly higher ($P < 0.01$) for the orally dosed birds fed the lactose diet than for the birds fed a similar diet containing glucose as the sole source of carbohydrate. The ^{28}Mg activity in the blood and femurs of birds given intraperitoneal injections of the isotope was not significantly altered by source of carbohydrate fed, although the values were somewhat higher for birds fed lactose. Differences in hemoconcentration were clearly shown in these studies. Hematocrit percentages were significantly higher ($P < 0.01$) in birds fed the diet containing lactose as compared with birds not receiving this sugar in their diet.

DISCUSSION

As observed in these studies, the type of carbohydrate fed did not greatly affect the absorption of orally administered doses of ^{28}Mg as measured by the retention of this isotope in the chick by a whole-body counter over a 48-hour period (trial 1) or a 4-hour period (trial 2). In view of the existing evidence which has shown that lactose enhances the absorption of several alkaline earth cations in rats (2–6), the results of these studies with chicks do not rule out a similar effect because magnesium absorption was not measured from isolated gastrointestinal segments. Despite the apparent lack of influence on absorption or retention or both as measured by the whole-body counter, ^{28}Mg in the blood and bone was significantly higher in birds fed the diet containing 25% lactose in which the isotope was administered orally.

The results of previous studies (1) concerned with the influence of carbohydrate source on magnesium metabolism in the chick indicated that birds fed diets containing lactose consumed significantly greater amounts of feed than their counterparts fed identical diets differing only in carbohydrate source. Feed consumption would, therefore, be a major factor in explaining differences in the plasma magnesium levels since birds consuming the lactose diets would have more magnesium available for absorption. Differences in feed consumption, however, would not be expected to markedly influence the amount of ^{28}Mg absorbed from a single oral administration.

The increased concentration of the orally administered isotope in the blood of chicks fed the diet containing lactose over that observed in chicks fed the diet containing only glucose as a carbohydrate source was shown to be significant only at the initial withdrawal of blood (trial 1, 8 hours post-dosage; trial 2, 4 hours post-dosage). A portion of the differences in blood ^{28}Mg activity may be attributed to hemoconcentration. The increased levels of ^{28}Mg in the blood, however, cannot be attributed entirely to hemoconcentration since the differences observed between chicks fed the 2 diets were considerably greater when the isotope was administered orally than when

it was administered by intraperitoneal injection.

The uptake of orally administered doses of ^{28}Mg into the bone was also increased by the inclusion of lactose in the diet. Aikawa et al. (9) have reported a rapid plasma clearance of parenterally administered doses of ^{28}Mg in rabbits; verification of this effect was shown by an accumulation of this isotope in the bone within 2 hours. Differences in femur radioactivity between birds fed the glucose and lactose diets were observed in trial 1 at 48 hours post-administration even though blood ^{28}Mg was not statistically different at the 16-, 24-, or 48-hour intervals. The increased amounts of ^{28}Mg in the femurs of lactose-fed chicks may have resulted from deposition at earlier time intervals following isotope administration. The increased levels of injected ^{28}Mg deposited in the femurs of chicks fed the lactose diet were not statistically different from those of chicks fed the glucose diet. Elevated femur levels of isotope administered by intraperitoneal injections would be difficult to explain in view of the results of studies with rats (4) which showed that lactose did not affect the utilization of calcium once this mineral had gained access to the animal body. Lengemann (4) also indicated that injected doses of lactose would not increase the uptake or removal of ^{45}Ca or ^{85}Sr to or from the bone over a 2-week period. In similar studies with rabbits, Aikawa (10) reported that simultaneous injections of ^{28}Mg and insulin, glucose or both, resulted in only slight increases in ^{28}Mg content of the bone, liver, and skin. Wasserman et al. (6) have also studied the effect of lactose on ^{45}Ca metabolism in the chick and in the rat and observed that this sugar did not increase the gastrointestinal absorption of ^{45}Ca in the chick as it did in the rat.

The influence of lactose in a diet which is deficient or marginally adequate in magnesium on the chick's utilization of magnesium appears to be related to three major factors. 1) Lactose is poorly metabolized by the chick and results in a diarrhetic condition at the level fed in these studies. Since the chick derives little energy from the lactose, more feed is required for the chick to obtain equal amounts of energy intake as compared with chicks fed diets containing a more utilizable carbohydrate such as glucose. Nutritional deficiencies, in this case a magnesium deficiency, may then be depressed because of increased feed (and hence magnesium) consumption. The work of Waibel and Mraz (11) showed that the inclusion of 20% lactose in a chick's diet low in vitamin D resulted in increased growth and bone ash when compared with chicks fed a diet not containing this sugar. 2) The inclusion of lactose in a chick's diet has been shown in these studies to exert an effect on hemoconcentration which may be partially responsible for the increased plasma levels of magnesium of chicks fed lactose in their diets. 3) An effect of lactose in promoting better or more efficient absorption of magnesium cannot be overlooked since significantly higher amounts of radioactivity were noted in the blood and femurs of chicks fed lactose as compared with those fed a similar diet containing glucose as the sole source of carbohydrate.

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Selenium Involvement in the Oxidation by Rat Liver Tissue of Certain Tricarboxylic Acid Cycle Intermediates [1,2]

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ABSTRACT In 3 separate experiments, rats were fed for 5 or 11 weeks a basal diet deficient in selenium and vitamin E, or similar diets supplemented with either or both of these factors and ethoxyquin. On termination of the feeding period the rats were killed, their livers removed and subjected to studies in vitro. Results showed that selenium was associated with the oxidation of pyruvate by the liver preparations, but not with oxidation of succinate. When the feeding period was extended from 5 to 11 weeks, it was necessary to add vitamin E to the basal diet in order for the effect of selenium on pyruvate metabolism to be manifested. The co-involvement of selenium and vitamin E was evident even in the presence of presumably adequate amounts of the synthetic antioxidant, ethoxyquin. The data suggest a direct involvement for selenium in the oxidative processes of the tricarboxylic acid cycle, and further suggest a metabolic role for vitamin E over and above that of a lipid antioxidant.

A specific metabolic function for selenium has not been demonstrated in animal tissues even though it appears to have an essential function in the prevention of certain metabolic disorders. Examples of these are liver necrosis in the rat (1), certain myopathies such as white muscle disease in lambs and calves (2) and exudative diathesis of chicks (3–4), all of which respond to dietary supplementation with 0.1 ppm selenium. Since vitamin E has also been shown to exert some influence upon these same dystrophic conditions, the possibility that both provide the essential biological function as in vivo lipid antioxidants has been postulated. Lowered lipid autoxidation, in early stages, has been related by Bieri (5) to dietary supplementation with 0.33 ppm selenium as evidenced by significant reduction in the thiobarbituric acid (TBA) value of the liver, kidney and heart, compared with a selenium-deficient group.

Although selenium has been shown to possess the properties of an in vivo lipid antioxidant (5–8), it appears to have further metabolic roles, because when sheep or cattle are fed diets of legume hay grown on low selenium soils, the offspring develop white muscle disease (9) although legumes are potentially rich sources of the naturally occurring antioxidant — vitamin E (10–11). Supplementation of such diets with 0.1 ppm Se (2) or parenteral administration of 20 mg Na_2SeO_3 to the pregnant ewe (9) completely prevented white muscle disease in the young, whereas further supplementation of the ewe's diet with additional vitamin E did not prevent the condition (2).

In the present investigation the involvement of selenium was studied in certain oxidative processes of the tricarboxylic acid cycle. Liver preparations from rats receiving diets differing in their content of selenium and certain lipid antioxidants were used to measure the effects of selenium or the antioxidants upon the oxidative rates of certain citric acid cycle intermediates.

EXPERIMENTAL PROCEDURE

A series of 3 experiments was conducted with male rats of the Wistar strain, fed various modifications of a low selenium, 30% Torula yeast diet shown previously to produce necrosis (12). The first trial compared the oxidation of pyruvate or succinate by liver preparations

Received for publication September 1, 1966.
[1] Technical Paper no. 2085, Oregon Agricultural Experiment Station.
[2] This study was aided by grants from the Muscular Dystrophy Associations of America, Inc., and from the Selenium-Tellurium Development Association.

TABLE 1

Experimental design for dietary treatments

	No. animals/group, trial no.		
	1	2	3
Basal	10(10) [1]		10(2)
Basal + ethoxyquin		10(10)	10(7)
Basal + vitamin E	10(10)		10(10)
Basal + ethoxyquin + vitamin E		10(10)	10(9)
Basal + selenium	10(10)		10(8)
Basal + ethoxyquin + selenium		10(9)	10(9)
Basal + vitamin E + selenium	10(10)		10(10)
Basal + ethoxyquin + vitamin E + selenium		10(10)	10(9)

[1] Numbers in parentheses indicate number of animals completing the experiment.

TABLE 2

Composition of rat diets

	Basal	Basal + vitamin E	Basal + selenium	Basal + vitamin E + Se
Torula yeast, g	300	300	300	300
Cornstarch, g	574	574	574	574
Glucose, g	6	6	6	6
Salts,[1] g	40	40	40	40
B-vitamin mix,[2] g	10	10	10	10
Cellulose,[3] g	20	20	20	20
Stripped lard,[4] g	50	50	50	50
Methionine,[5] g	1.9	1.9	1.9	1.9
Ethoxyquin,[6] g	0.125	0.125	0.125	0.125
Na_2SeO_3,[7] g	—	—	0.00213	0.00213
Vitamin A, IU	2000	2000	2000	2000
Vitamin E,[8] IU	—	500	—	500
Vitamin D, IU	555	555	555	555

[1] Jones and Foster (18).
[2] B-vitamin mix consisted of: (in milligrams) thiamine·HCl, 0.6; riboflavin, 1.2; pyridoxine, 0.4; niacin, 5.0; Ca pantothenate, 4.0; inositol, 100.0; choline chloride, 200; *p*-aminobenzoic acid, 2.5; biotin, 1.0; folic acid, 1.0; and cyanocobalamin, 1, with sufficient starch to make 1 g.
[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[4] Obtained from Distillation Products Industries, Rochester, New York.
[5] Methionine was not included in the diets in trial 1.
[6] Ethoxyquin was not included in the diets of trial 1 nor of the control groups in trial 3.
[7] Equivalent to 1 ppm selenium.
[8] Vitamin E added as *dl*-α-tocopheryl succinate (obtained from Distillation Products Industries).

from rats that had received different dietary levels of selenium and vitamin E. Additions of selenium and vitamin E were made to the in vitro liver preparations to determine whether an active product could be formed during this incubation period. In the second trial, an investigation into a more specific involvement of selenium in the oxidative pathways dependent upon either NAD (pyruvate) or flavoprotein (succinate) was carried out by isolating these pathways through enzyme inhibitors in rat liver mitochondria. The test animals received adequate lipid antioxidant protection (ethoxyquin)[3] in their diets in addition to different dietary supplementation levels of selenium and vitamin E. The third trial related the 2 proposed functions of selenium to its lipid antioxidant properties and to its more specific role in an oxidative pathway by combining treatments from the previous 2 trials. The experimental design for the dietary treatments is shown in table 1 and the composition of the diets is outlined in table 2.

After feeding for 5 weeks in trial 1 or 9 weeks (in addition to 2-week pretrial with a deficient diet without ethoxyquin) in trials 2 and 3, the rats were removed for metabolic studies in vitro. They were killed by a blow on the head, and the

[3] 1,2-Dehydro-6-ethoxy-2,2,4-trimethylquinoline, Monsanto Company, St. Louis.

livers were removed immediately and placed in ice-cold saline. In trial 1, a 4-g sample of each liver was weighed into 40 ml of cold 0.25 M sucrose solution at pH 7.4 containing 0.0001 M EDTA and 100 ml of 0.1 M Krebs-Ringer phosphate buffer. The whole preparation was then homogenized in a Potter-Elvehjem homogenizer and cell wall and connective tissue were removed by centrifuging at $600 \times g$ for 10 minutes at 1 to 3°. The utilization of substrate (succinate or pyruvate) by the homogenate was indicated through oxygen consumption values measured in a Warburg apparatus following the procedures of Umbreit et al. (13). Each reaction flask contained 300 μmoles NaCl, 12 μmoles KCl and 4 μmoles of $MgSO_4 \cdot H_2O$, adjusted to pH 7.4. Just prior to each run, 40 μmoles of sodium phosphate buffer at pH 7.4 were added after gassing with oxygen for approximately 20 minutes while in an ice bath. Sixty micromoles of either pyruvate or succinate were added to each flask along with 3 μmoles of NAD and 0.5 ml of homogenate (equivalent to 0.05 g of the original liver).

In trial 2 and 3 liver mitochondria were isolated by homogenizing in a medium containing 3×10^{-1} M sucrose, 5×10^{-2} M Tris, 1×10^{-4} M $EDTA \cdot Na_2$, 1×10^{-4} M $MgCl_2 \cdot 6H_2O$, 5×10^{-3} M Na pyruvate, 3×10^{-5} M Na fumarate and 0.3 g albumin [4] made up to 250 ml final volume. After homogenization the slurry was centrifuged for 10 minutes at $400 \times g$ at 1 to 3°. The supernatant was carefully decanted and centrifuged for 10 minutes at $9000 \times g$. The supernatant was discarded, and the mitochondrial "button" was resuspended in the same medium used in homogenizing except the concentration of $EDTA \cdot Na_2$ was changed to 3.1×10^{-3} M. The suspension was again centrifuged at the same speed, the supernatant discarded, and a new suspension medium used for final preparation for the incubation studies. The mitochondria were incubated in a medium containing 30 mg bovine serum albumin, 100 KM units type II hexokinase,[5] 10 μmoles adenosine 5′-triphosphate (ATP), 60 μmoles Tris buffer, 300 μmoles sucrose, 20 μmoles glucose, 20 μmoles $MgCl_2 \cdot 6H_2O$ and 100 μmoles of pH 7.4 potassium phosphate buffer. In addition, either 18 μmoles of Na succinate or Na pyruvate were included in the incubation medium along with 1 ml of the mitochondrial preparation (equivalent to 0.1 g of the original liver). Depending upon the in vitro treatment the following were included: 1.0 μmole NAD, 1.8 μmoles Amytal,[6] 20.0 μmoles Na malonate and 2.0 μmoles Na fumarate.

In vitro additions of selenium, vitamin E or ethoxyquin were administered directly to the liver preparations and rehomogenized. The addition of 40 μg *dl*-α-tocopherol and ethoxyquin per ml of homogenate was made with absolute ethanol as the carrier. In all other treatments which did not include either vitamin E or ethoxyquin, equivalent amounts (0.001 ml/ml of homogenate) of ethanol were used. Selenium was added in the concentration of 0.6 mg Se/ml of homogenates as Na_2SeO_3.

The statistical analysis of the data was conducted on a randomized split-split plot design analysis of variance.

RESULTS

Incubation in vitro of liver homogenates with either pyruvate or succinate separates the oxidative processes into NAD-dependent (pyruvate) or flavoprotein-dependent (succinate) systems. The oxidation rates of these substrates by liver tissue from rats fed diets differing in selenium, vitamin E and content of a synthetic lipid antioxidant (when considered with various in vitro treatments) allows some speculation as to the metabolic function of selenium in these pathways.

Studies using pyruvate as the substrate.

A significant increase ($P < 0.01$) in the oxidation of pyruvate was noted by the groups receiving added dietary selenium as compared with the non-selenium supplemented groups in trial 1 (table 3). In the subsequent trial selenium supplementation again exerted a significant increase ($P < 0.01$, table 4) on the oxidation rates but only in the group receiving the combined supplement of selenium and vitamin E. The inclusion of 0.0125% ethoxyquin

[4] Albumin from bovine serum, crystallized and lyophilized, Sigma Chemical Company, St. Louis.
[5] Sigma Chemical Company.
[6] Amobarbital, Eli Lilly and Company, Indianapolis.

TABLE 3

Oxygen uptake of liver homogenates at intervals during incubation with sodium pyruvate substrate — trial 1

In vitro supplementation	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
None	0–30	12.6	14.9	14.7	16.8
	30–60	8.2	10.1	10.9	11.4
	60–90	6.1	7.5	8.9	8.8
Total		26.9	32.5	34.5	37.0
Vitamin E	0–30	11.3	15.6	15.4	17.5
	30–60	8.7	11.6	11.2	13.2
	60–90	7.3	8.0	9.8	9.7
Total		27.3	35.2	36.4	40.4
Se	0–30	8.6	12.6	10.2	14.5
	30–60	6.9	8.0	7.8	10.5
	60–90	6.9	6.5	5.3	7.1
Total		22.4	27.1	23.3	32.1
Vitamin E + Se	0–30	10.1	15.7	13.6	16.4
	30–60	6.1	9.0	9.5	11.2
	60–90	6.6	6.3	8.0	9.0
Total		22.8	31.0	31.1	38.6

TABLE 4

Oxygen uptake of liver mitochondria at intervals during incubation with sodium pyruvate substrate — trial 2

In vitro supplementation	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
None	0–30	25.9	22.6	23.2	27.9
	30–60	24.3	23.1	23.7	27.0
	60–90	21.0	20.1	21.6	24.7
Total		71.2	65.8	68.5	79.6
Na malonate	0–30	17.8	15.8	17.9	18.0
	30–60	13.7	13.9	14.6	15.8
	60–90	10.5	10.0	12.0	12.1
Total		42.0	39.7	44.5	45.9
Na malonate + vitamin E	0–30	15.5	14.3	15.7	15.8
	30–60	10.2	10.6	12.6	11.9
	60–90	9.1	7.7	9.1	9.8
Total		34.8	32.6	37.4	37.5

in the selenium-supplemented diets to give adequate antioxidant protection did not nullify the response due to selenium and vitamin E suggesting that their activities are based on properties other than those of a general lipid antioxidant. Moreover, further inclusion within the incubation medium of additional antioxidant protection

— vitamin E or ethoxyquin — failed to give an additional increase in the oxidation rate. Some of the response noted by in vitro additions of vitamin E during the first trial must have been a direct result of the antioxidant protection which was not adequately supplied in the diet. A large decrease was noted in the oxidation rate during the first trial which was markedly reduced in the later trial especially by the in vitro inclusion of the succinic dehydrogenase inhibitor, malonic acid. In such case the oxidation levels were maintained for the first 60 minutes of incubation and only decreased slightly during the final 30 minutes of incubation.

As noted in trial 1, either dietary supplementation with selenium or vitamin E significantly increased the oxidation of pyruvate ($P < 0.01$), but the statistical test for interaction between selenium and vitamin E was not significant. The extension of the feeding trial from 5 weeks, as used in the first trial, to 11 weeks as used in the final 2 trials allowed a sufficient time for adequate depletion of the body stores of vitamin E to study its relationship to selenium function. The administration of either selenium or vitamin E singly did not significantly alter the rate of oxidation of pyruvate by the mitochondria (table 4). However, the combined dietary supplementation with selenium and vitamin E resulted in a significant increase ($P < 0.01$) in the oxidation rate and a significant interaction ($P < 0.05$) was noted between selenium and vitamin E. This was an immediate response that occurred within the first 30 minutes of incubation and continued throughout the total 90-minute period. The antioxidant protection was apparently sufficient, since further in vitro addition of antioxidants — vitamin E or ethoxyquin — did not further increase the oxidation rate.

Selenium added to the homogenates in vitro at the levels used proved to be toxic. This was particularly evident in preparations from the group also receiving dietary selenium, where the oxidation levels were greatly reduced. The administration of vitamin E either in the diet or in vitro tended to alleviate some of the toxic properties of the selenium in vitro.

The inclusion of a succinic dehydrogenase inhibitor, sodium malonate, to limit oxidative measurements to those dependent upon NAD as the cofactor, decreased the oxygen uptake values and also eliminated the significant interaction between selenium and vitamin E on pyruvate utilization. Similar results were observed in trial 3 although dietary selenium tended to increase the oxidative rate to a level approaching statistical significance (table 5). Further analysis of the problem suggested that blocking the citric acid cycle with sodium malonate may have limited the available supply of oxaloacetic acid, and that this was responsible for the nonsignificant differences observed from selenium and vitamin E supplementation. This conjecture was substantiated by inclusion of oxaloacetic acid along with sodium malonate in some preparations, in which cases the oxidative potential of the system was increased (table 6).

Addition in vitro of vitamin E along with the inhibitor did not increase the oxidation of pyruvate by the mitochondrial preparations and generally decreased the oxidation rates. The limitation of adequate oxaloacetic acid described above may also have been a factor in this case.

Studies using succinate as the substrate. The utilization of succinate was not altered by administration of selenium in the diet (table 7). This was observed in all 3 trials, indicating that selenium must not be directly involved in the oxidation of succinate by the liver preparations. Moreover, in trials 2 and 3 (tables 8 and 9) after the inclusion of Amytal to isolate the oxidation of the succinate-dependent system no differences were observed as the result of dietary supplementation. The slight increase in oxygen consumption noted, when Amytal was not present, by the group receiving dietary selenium could thus be attributed to oxidative pathways other than those involved in the oxidation of succinate.

Although a significant increase ($P < 0.01$) in the oxygen consumption values by the liver homogenates from the rats receiving dietary vitamin E was noted in trial 1, further supplementation with in vitro vitamin E did not increase succinate oxidation except by the groups receiving

TABLE 5

Oxygen uptake of liver mitochondria at intervals during incubation with sodium pyruvate substrate — trial 3

In vitro supplementation	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
		Without ethoxyquin			
Na malonate	0–30	6.9	7.8	6.6	6.1
	30–60	6.9	6.9	6.3	6.5
	60–90	5.7	6.5	6.3	7.3
Total		19.5	21.2	19.2	19.9
Na malonate + vitamin E	0–30	6.7	8.5	6.8	7.3
	30–60	4.3	7.2	5.6	6.1
	60–90	2.9	6.6	5.4	6.3
Total		13.9	22.3	17.8	19.7
Na malonate + ethoxyquin	0–30	6.5	7.9	7.4	6.5
	30–60	4.5	6.6	5.6	6.2
	60–90	3.5	5.5	5.1	6.7
Total		14.5	20.0	18.1	19.4
		With ethoxyquin			
Na malonate	0–30	7.2	5.1	6.7	6.8
	30–60	7.3	6.3	5.9	7.3
	60–90	5.9	6.5	6.6	6.9
Total		20.4	17.9	19.2	21.0
Na malonate + vitamin E	0–30	8.2	6.9	6.8	6.7
	30–60	6.9	6.4	6.6	5.7
	60–90	5.1	5.9	5.9	5.9
Total		20.2	19.2	19.3	18.3
Na malonate + ethoxyquin	0–30	6.9	6.6	7.6	6.8
	30–60	6.6	6.1	5.9	5.8
	60–90	4.8	5.7	6.0	5.9
Total		18.3	18.4	19.5	18.5

TABLE 6

Influence of oxaloacetic acid and sodium malonate on oxygen uptake with sodium pyruvate by liver mitochondria from normal rats

Incubation time	In vitro addition			
	None	Oxaloacetic acid	Oxaloacetic + malonate	Malonate
minutes	*μliters*	*μliters*	*μliters*	*μliters*
0–30	14.1	14.5	13.3	9.3
30–60	7.8	13.9	13.2	6.1
60–90	5.5	11.3	9.7	5.1
Total	27.4	39.7 [1]	36.2 [1]	20.5

[1] Significantly different ($P < 0.01$) from treatments not marked within the same category.

TABLE 7
Oxygen uptake of liver homogenates at intervals during incubation with sodium succinate substrate — trial 1

In vitro supplementation	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
None	0–30	50.5	57.2	55.6	61.8
	30–60	37.1	49.5	41.5	45.7
	60–90	34.8	37.9	34.2	41.6
Total		122.4	144.6	131.3	149.1
Vitamin E	0–30	47.1	53.6	54.2	59.8
	30–60	36.9	48.9	44.5	47.7
	60–90	35.7	39.9	37.0	46.4
Total		119.7	142.4	135.7	153.9
Se	0–30	47.9	54.5	51.8	59.4
	30–60	37.3	45.8	37.7	45.3
	60–90	31.1	38.0	34.2	39.8
Total		116.4	138.4	123.7	144.5
Vitamin E + Se	0–30	44.1	52.4	51.7	55.5
	30–60	34.7	45.2	39.1	43.8
	60–90	33.8	35.0	35.5	35.4
Total		112.6	132.6	126.3	134.7

TABLE 8
Oxygen uptake of liver mitochondria at intervals during incubation with sodium succinate substrate — trial 2

In vitro supplementation	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
NAD	0–30	90.2	83.0	84.2	88.7
	30–60	74.0	72.5	71.4	78.8
	60–90	60.9	61.9	56.9	64.4
Total		225.1	217.4	212.5	231.9
Amytal [1]	0–30	62.4	58.1	57.4	57.9
	30–60	30.2	30.8	30.2	31.2
	60–90	16.6	16.3	18.3	15.8
Total		109.2	105.2	105.9	104.9
NAD + Amytal	0–30	69.3	67.1	67.2	65.9
	30–60	34.4	34.9	32.8	33.9
	60–90	17.8	18.4	20.5	19.9
Total		121.5	120.4	120.5	119.7
NAD + Amytal + fumarate	0–30	67.8	63.8	62.4	63.9
	30–60	34.8	35.7	35.4	36.0
	60–90	19.0	19.3	20.1	21.1
Total		121.6	118.8	117.9	121.0
Vitamin E + NAD + Amytal + fumarate	0–30	62.5	59.5	56.7	57.2
	30–60	32.5	35.1	32.5	32.9
	60–90	20.2	19.9	20.5	22.4
Total		115.2	114.5	109.7	112.5

[1] Amobarbital, Eli Lilly and Company, Indianapolis.

TABLE 9

Oxygen uptake of liver mitochondria at intervals during incubation with sodium succinate substrate — trial 3

In vitro supplementation (includes Amytal) [1]	Incubation time	O_2 uptake, dietary supplementation			
		Basal	With vitamin E	With selenium	With vitamin E + Se
	minutes	*μliters*	*μliters*	*μliters*	*μliters*
		Without ethoxyquin			
Succinate	0–30	55.3	56.4	53.9	52.6
	30–60	37.7	35.0	34.0	36.5
	60–90	30.1	28.5	28.4	28.9
Total		123.1	119.9	116.3	118.0
Succinate + vitamin E	0–30	41.7	53.9	51.2	49.3
	30–60	26.2	34.7	32.8	33.5
	60–90	19.4	26.7	27.0	26.9
Total		87.3	115.3	111.0	109.7
Succinate + ethoxyquin	0–30	52.2	56.2	53.4	49.5
	30–60	36.3	34.6	33.9	34.7
	60–90	27.5	27.1	27.4	27.8
Total		116.0	117.9	114.7	112.0
		With ethoxyquin			
Succinate	0–30	54.0	47.8	52.9	48.9
	30–60	35.4	34.7	34.2	33.3
	60–90	29.1	28.0	29.3	26.5
Total		118.5	110.5	116.4	108.7
Succinate + vitamin E	0–30	52.7	47.7	50.6	50.2
	30–60	34.1	34.5	32.8	32.1
	60–90	27.3	27.4	27.7	27.0
Total		114.1	109.6	111.1	108.3
Succinate + ethoxyquin	0–30	55.1	49.2	50.2	48.5
	30–60	34.8	35.6	34.3	32.9
	60–90	28.8	28.0	28.4	26.9
Total		118.6	112.8	112.9	108.3

[1] Amobarbital, Eli Lilly and Company.

dietary selenium. Vitamin E was not directly concerned in this oxidation pathway, since in trials 2 and 3 both selenium and vitamin E did not affect the oxidation of succinate. The addition of ethoxyquin in the diets of all rats in trial 2 would reduce the need for either vitamin E or selenium as lipid antioxidants. A slight but nonsignificant increase in oxidation of succinate by isolated mitochondria was noted for the group receiving dietary vitamin E and selenium when Amytal was not included in the incubation medium (table 8). By isolating the oxidation to succinate by including Amytal, the oxidation values for all dietary treatments were essentially equal. The addition in vitro of 3 μmoles NAD along with Amytal indicates that the level of Amytal used was not sufficient to inhibit all NAD-dependent oxidation. Through the inclusion of Amytal in the incubation medium the buildup of fumarate appears not to be a serious problem since no further decrease in oxidation was noted by the inclusion of in vitro fumarate (table 8).

In trial 3 the importance of a lipid antioxidant (ethoxyquin) was studied on the mitochondrial oxidation of succinate in preparations from rats receiving different dietary levels of selenium and vitamin E. As shown in table 9, succinate oxidation from the ethoxyquin-treated groups was not significantly different from those not receiving dietary ethoxyquin. As in trial 2 the incubation medium contained Amytal to isolate the succinate oxidation pathway. Provision of further antioxidant protection by addition of either vitamin E or ethoxyquin did not significantly alter the oxidation rates by the mitochondria. Oxygen uptakes from the various dietary levels of selenium and vitamin E were not significantly different, thus confirming the results in trial 2 that selenium and vitamin E are not directly involved in the oxidation of succinate by liver mitochondria.

DISCUSSION

These data suggest that selenium and probably vitamin E are involved in the oxidation of pyruvate by rat liver tissue. This phenomena with animals adequately protected from lipid autoxidation damage by the presence of ethoxyquin in their diets suggests that this metabolic activity would presumably be one closely associated with pyruvate oxidation. Adequate antioxidant protection is indicated by the data, since the respiratory rates were maintained during the 90-minute incubation period and further addition of antioxidant protection by in vitro supplements of vitamin E or ethoxyquin did not further increase the oxidative rates. Similar effectiveness of vitamin E, DPPD, and ethoxyquin on prevention of respiratory decline during incubation of succinate or α-ketoglutarate has been reported by Corwin and Schwarz (12). Krishnamurthy and Bieri (14) indicate that certain lipid antioxidants like BHT are ineffective in the prevention of vitamin E-deficiency symptoms because of the poor availability of these substances to the proper tissue or cellular structure requiring antioxidant protection. However, with adequate antioxidant protection provided by ethoxyquin in this study the differences observed due to selenium and vitamin E were in addition to their reported antioxidant properties (5–7, 15).

Evidence of selenium function in the α-ketoglutarate oxidase system was reported by Connolly and Schwarz [7] with prenecrotic liver mitochondria from rats fed deficient diets of selenium and vitamin E for 14 days. Increased oxidase activity was noted in the selenium-supplemented group with a 30% greater reduction of 2,6-dichlorophenol indophenol over the deficient group. Later, Connolly and Schwarz [8] observed a 25% increase in the $^{14}CO_2$ release from labeled α-ketoglutarate by the selenium-supplemented group when the succinic oxidase system was inhibited with malonate. Certain similarities exist between the oxidative pathways involving α-ketoglutarate and pyruvate with the same co-factors being required.

In this study selenium was not associated with the oxidation of succinate by the rat liver preparations since variations in dietary selenium did not result in significant differences. Vitamin E in the first trial had a marked effect on succinate oxidation and respiratory decline, but in the subsequent trials when antioxidant protection was provided, vitamin E was shown not to have a direct role in succinate oxidation. A report by Corwin and Schwarz (12), indicates that α-tocopherol is associated with the regulation of succinate oxidation by preventing a decline in the oxidation rate when inadequate vitamin E protection was provided. Corwin (16) noted that the inclusion of Amytal in the incubation medium eliminates the vitamin E response during succinate oxidation, thus suggesting that vitamin E activity is dependent upon NAD. He observed that when the incubation medium included NAD with the succinate, a marked inhibition of oxygen utilization by vitamin E-deficient mitochondria occurred, whereas the addition of vitamin E reduced this inhibition. Results of this trial substantiate Corwin's observations with respect to the effect of Amytal; however, the inhibitory effect of NAD was not evident. It is possible that the involvement of NAD

[7] Connolly, J. D., and K. Schwarz 1963 Effect of vitamin E, selenium and methionine on α-ketoglutarate oxidase activity of rat liver mitochondria. Federation Proc., 22: 652 (abstract).

[8] Connolly, J. D., and K. Schwarz 1965 Effect of selenium and vitamin E on α-ketoglutarate utilization by liver mitochondria. Federation Proc., 24: 623 (abstract).

on succinate oxidation was lessened by the inclusion of Amytal but that there were still adequate levels for the NAD-dependent systems.

Since selenium and vitamin E activity was associated with the oxidation of pyruvate but not succinate, the location of metabolic activity appears not to involve the cytochrome system directly. Equal involvement of both substrates would have been expected if vitamin E and selenium were acting on the cytochrome system.

The interaction between selenium and vitamin E points to their interdependence in a more specific metabolic role. As indicated in trial 2 selenium or vitamin E were ineffective on the oxidation of pyruvate until both were included in the same diet. There was an immediate response to the joint supplementation in oxidation values which continued throughout the total 90-minute incubation period. The necessity for a long feeding period in selenium and vitamin E studies was demonstrated because the 5 weeks used in trial 1 apparently were not adequate to deplete the body stores of vitamin E to the point where this interdependence was evident. It has been suggested by Desai and Scott (17) that vitamin E may be carried by a selenolipoprotein fraction associated with serum γ-globulin, since selenium improves the retention of α-tocopherols, especially *d*-α-tocopherol.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to L. F. Remmert, formerly Department of Agricultural Chemistry, Oregon State University, and to K. E. Rowe, Department of Statistics and J. Kaufmes, Department of Animal Science, Oregon State University, for technical advice and assistance in conducting these studies.

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Some Effects of Caloric Restriction and Deprivation on the Obese Hyperlipemic Rat [1]

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ABSTRACT To explore the nature of the genetic obesity in rats of the obese hyperlipemic strain (designated as "fatties"), we studied rates of deposition and of depletion of adipose tissue stores under varying conditions of food intake and stage of growth. When totally starved, young fatties survived until all visible fat stores were exhausted; their low water consumption indicated that they were metabolizing principally fat. Control non-obese rats died much earlier, after exhausting their much smaller fat stores. Controls receiving a diet of fat had survival rates comparable to those of fatties. Terminal weights of all rats were comparable. Adult fatties showed no abnormalities in response to 1, 2 or 7 days of fasting. Young fatties pair-fed to normal sibs for 14 days, with a 40% reduction in weight gain, grew abnormally in that they laid down more fat, and less muscle and bone, than the controls. These fatties finished the experiment as obese as they were at the start. Fat, muscle and bone gains were estimated from gains in representative fat pads and muscle and from the increase in length of a long bone.

Rats homozygous for the mutant gene "fatty" (*fa*) are obese and hyperlipemic, and are designated as "fatties" (1). Their food intake is excessive (2). In preliminary observations, total starvation caused eventual disappearance of all visible fat stores, but reduction in food intake during the period of growth and development of the obesity produced rats which appeared to be obese but probably undersized. The present study was undertaken to verify these indications that the fatty diverts from growth to fat storage an abnormally great proportion of a limited caloric intake. In addition, the effect of caloric restriction on the characteristic liver hypertrophy was studied.

EXPERIMENTAL

Three experiments were carried out: total starvation in young and adult rats and food restriction in young rats.

Starvation in young rats. Starving fatties were compared both with starving non-obese controls, and with non-obese controls receiving cottonseed oil ad libitum, but no other food. In both comparisons initial body weights were approximately matched so that caloric requirements would be similar initially. Three fatty females and 3 normal sib males were starved from the age of 60 days, when their weights were about the same. An additional pair was autopsied at this time for various tissue and organ measurements. After 7 days of starvation one pair was autopsied. After 11 days of starvation the fatty females weighed about the same as normal sib females that had been full-fed to this point; accordingly the 4 normal females were started with the cottonseed oil diet at age 71 days. All rats had water ad libitum. Weight curves and survival times were observed, also status of adipose tissue at death and during the experiment. A few observations were also made on water and oil consumption.

Starvation in adults. Groups of adult fatties and non-obese controls (usually sibs) were killed while full-fed, and after fasting 18, 42 and 136 hours. There were generally 8 pairs of each sex in each group; the average age of each set was 40 weeks, individual ages ranging from 25 to 77. All rats were initially in good physical condition, not losing weight. Observations include body weights, fatness, organ weights, serum lipids. Other observations on these rats will be published elsewhere as part of a study of insulin status in fatties.

Received for publication September 6, 1966.

[1] This research was supported in part by Public Health Service Research Grant no. AM 08272, and Special Fellowship K3-AM-22,507, both from the National Institute of Arthritis and Metabolic Diseases.

Food restriction in young rats. Six young (27 to 30 days) male fatties were pair-fed to normal male sibs for 14 days and were then autopsied to determine fatness and various other tissue and organ weights. A control series, killed at 29 to 30 days of age, supplied the tissue and organ weights pertinent to the beginning of the experiment; there were 12 fatties and 24 non-obese controls, all contemporaries of the 6 experimental pairs, a few from the same litters.

Observations at autopsy. The body was thoroughly drained of blood by cutting the heart of the anesthetized animal. Liver, fat pads and muscle were weighed, and tibia length was measured. Serum lipids and protein were determined (see below for methods). The length of the tibia was used as a measure of skeletal size; the cube of this length is dimensionally comparable to weight and increases at rates comparable to that of body weight. To assess fatness we used the weight of all abdominal adipose tissue which remains after removal of the gut with its supporting tissues, but excluding from measurement any fat tissue lying along the edge of the diaphragm and around the xyphoid cartilage. Muscle mass was represented by the weight of muscle remaining around tibia and fibula after muscle attached to the heel tendon had been stripped away and discarded.

Diets. Except for the experimental periods, rats were fed our stock diet, of which a description follows. Lactating mothers, and young to 7 weeks of age, received ad libitum both a commercial pelleted rat diet[2], based on soybean meal, fish, skim milk, corn, wheat and corn oil, with about 6% of fat and 20% of protein, and our lactation diet, consisting of the following per 100 g diet: (in grams) wheat, 45; dried skim milk, 35; lactalbumin, 10; cottonseed oil, 5; yeast, 2.5; bone ash, 1.6; NaCl, 0.5; $MgSO_4$, 0.3; (in milligrams) $FeSO_4$, 52; $MnSO_4 \cdot H_2O$, 15; ZnO, 1; NaF, 0.75; Cu_2O, 0.45; $KAl(SO_4)_2 \cdot 12H_2O$, 0.35; $Na_2MoO_4 \cdot 2H_2O$, 0.05; $CoSO_4 \cdot 7H_2O$, 0.10; borax, 0.175; vitamin B_1, 0.5; vitamin B_6, 0.5; vitamin B_2, 1; niacinamide, 2; Ca pantothenate, 2; menadione, 0.25; pteroylglutamic acid, 0.125; vitamin B_{12}, 0.001; α-tocopherol, 1.5; α-tocopheryl acetate, 1.5; and carotene concentrate supplying 750 units vitamin A activity. From 7 weeks of age, stock rats received the pelleted diet, and spinach once a week. Young rats were weaned at 3.5 weeks. During the 14 days of food restriction we used diet L15, made by the addition of 15 parts cottonseed oil and 5 parts lactalbumin to 80 parts of the lactation diet; it thus contained about 20% fat.

Measurement of food consumption. Fatties receiving a restricted intake spill much less food than full-fed controls; hence measurement of, and compensation for, spillage is of great importance. All our rats, including these, were housed in cages with wire-mesh floors, suspended over excreta pans; for this work the pans were lined with absorbent paper which was changed daily. The food cup used was a can with top cut off at about 30° to the horizontal. This cup was firmly attached in a larger enclosure of light sheet metal, having a bottom, and also sides and a back reaching to the top of the cage. The dimensions of this enclosure are such as to prevent the rat from entering far enough, or turning around, so as to deposit excreta within the enclosure. Fastened to the top of the can and the 3 sides of the enclosure is a piece of wire mesh with a central hole big enough for the rat to eat through it. Most food spillage falls through this mesh to the floor of the enclosure. The sides and back are removable; the remaining assembly is light enough to be weighed as a whole. All urine and feces fall into the excreta pan under the cage, together with a light scattering of food. Water drip is caught in a separate small pan under the tip of the bottle. Spillage and food consumption were determined daily at 11 to 11:30 AM, and the restricted rats were fed at this time.

Serum lipids and protein. Total serum lipids were determined by their light-scattering power when diluted in physiological saline. We used a Turner fluorometer no. 110, with blue lamp, primary filters Wratter 58 (wave lentgh 525 mμ) and 2ND (neutral, 1% transmission), and no secondary filter. As permanent and reproducible nephelometric standard we used a

[2] Hemlock Hollow, obtained from GLF-Agway, Waverley, New York.

suspension of Pyrex glass, 92 ppm, prepared according to Anderson and Engle (3). Readings were taken before and after adding 50 μliters serum to 1 ml 0.9% NaCl in a 10 × 75 mm tube; the light paths were suitably masked to allow the use of this small volume. The results are expressed as percentage glass equivalent calculated for undiluted serum, and these values are in the same range as grams of lipid per 100 ml serum. In general, however, the light-scattering power resides in large, low-density lipoproteins and is principally affected by the triglyceride content of the serum (4). Serum protein was determined by the biuret reaction (5), using the same saline dilution prepared for the lipid determination.

RESULTS

The experiment on total starvation in young fatties is summarized in figure 1. The starved non-obese controls lost weight rapidly and lived only 8 days. The starved fatties, and the controls fed cottonseed oil ad libitum, lost weight at similar and much slower rates and died at approximately the same final weight. All rats were free of visible fat stores at death.

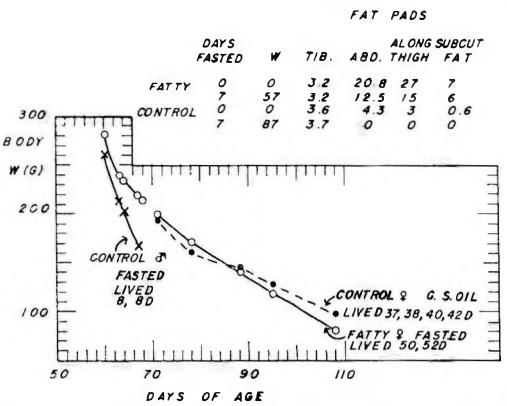

Fig. 1 Effect of fasting on body weight and survival in female fatties (open circle), and male controls of the same age, initial weight and litter origin (crosses). An additional female control group of the same age, initial weight and litter origin (solid circles), started later, received cottonseed oil ad libitum. Table shows *change* in body weight and actual weight of various fat pads after 7 days of fasting; each line represents one rat. *Tib.* = tibia length in centimeters. *Abd.* = retroperitoneal + epididymal or perimetrial fat pads. Subcutaneous fat was taken from neck and shoulders. All fat tissue values are in grams.

The final weights were probably in fairly close relation to the magnitude of the non-fat carcass, which would be similar in littermate females, whether fatty or not, and considerably greater in males from the same litters.

During the fifth to seventh days of fasting, water consumption by the fatties was 7 ml/day, and the starving controls drank 35 ml/day. The low figure for the fatties suggests a diet largely of fat, whereas the high figure for the normals is consistent with a terminal diet largely of protein, after fat stores have been practically exhausted. During the first week that rats were fed cottonseed oil, the average daily consumption was 5 ml; subsequently measurements were not made, but consumption appeared to decrease somewhat during the course of the experiment.

The data in the insert of figure 1 show adipose tissue weights in individual rats, both initially, and after 7 days of fasting. In 7 days the fatty lost more fat from the selected 3 tissues than the non-obese rat had to start with. The pattern of fat loss in the 2 fatties suggests that subcutaneous fat may be called upon later (i.e., may be less responsive to the fat-mobilizing stimulus of fasting) than the other more purely fat tissues. This idea is supported by the appearance of adult fatties raised with a restricted food intake; excessive subcutaneous fat, especially around the neck and shoulders, is a very prominent feature.

Table 1 shows the observations on fasting adults. Female fatties were relatively much fatter than male fatties, as indicated by the initial body weight ratios for paired, usually litter-matched, fatties and normals. This ratio was over 2 in females, only 1.5 in males. The degree of fatness is also shown by the ratio of abdominal fat tissue to the cube of tibia length. This experiment, unlike that shown in figure 1, involved only the earliest stages of fasting. Fat stores were only moderately depleted in the non-obese rats, and individual variation, which becomes very large in adult fatties, obscured the evidence of depletion of fat stores in the fatties. As we have reported previously (2, 4), overnight fasting did not significantly reduce the hyperlipemia of fatties. With additional fasting, serum lipids decreased, eventually after 7

TABLE 1

Effect of 1, 2 and 7 days of fasting on adult rats

Fasting period	Wt	Wt loss	Serum lipid	Calf muscle/ tibia length	Liver/ tibia length	Abdominal fat/ tibia length
hours	*g*	*g*	*%*	*mg/cm³*	*mg/cm³*	*mg/cm³*
			Normal rats			
			females			
Full-fed	294 ± 6.7		0.132 ± 0.020 [1]	27.1 ± 0.47	0.166 ± 0.006	0.184 ± 0.020
18		18	0.030 ± 0.002	25.1 ± 0.56	0.118 ± 0.003	0.184 ± 0.027
42		33	0.021 ± 0.004	24.8 ± 0.75	0.107 ± 0.003	0.152 ± 0.023
136 [2]		42				
			males			
Full-fed	518 ± 8.7		0.152 ± 0.032	29.7 ± 0.83	0.207 ± 0.014	0.182 ± 0.027
18		23	0.040 ± 0.004	29.0 ± 0.76	0.148 ± 0.007	0.168 ± 0.013
42		40				
136		87	0.028 ± 0.005	26.4 ± 0.68	0.114 ± 0.004	0.131 ± 0.024
			Fatties			
			females			
Full-fed	648 ± 19		1.82 ± 0.71	28.4 ± 0.46	0.40 ± 0.035	1.74 ± 0.167
18		20	1.83 ± 1.25	26.4 ± 0.42	0.37 ± 0.030	1.73 ± 0.123
42		44	0.53 ± 0.30	25.7 ± 0.81	0.30 ± 0.011	1.70 ± 0.135
136		68				
			males			
Full-fed	790 ± 18		0.66 ± 0.118	28.1 ± 1.0	0.45 ± 0.031	1.57 ± 0.174
18		29	0.40 ± 0.091	27.4 ± 0.75	0.33 ± 0.014	1.35 ± 0.129
42		50				
136		112	0.10 ± 0.009	25.6 ± 0.89	0.24 ± 0.012	1.26 ± 0.084

[1] Mean ± SE. There were about 30 rats in each weight group, comprising usually 7 to 9 rats full-fed, and the full-fed weights of the 7 to 9 rats in each fasted group. Average age per group was 40 ± 1 weeks; individual ages ranged from 25 to 76 weeks. Lipid and tissue measurements were not available for 4 of the groups.

[2] A nearly 7-day fast is well-tolerated by rats of this age and size. Weight loss in the normal rats was 14 to 17% of initial weight. The young growing rats of figure 1 were nearly moribund after 7 days of fasting, and had lost 35% of their initial weight.

days reaching the normal full-fed range. Calf muscle weight decreased gradually in all 4 groups, at approximately the same rates. Liver weight decreased in all groups at about the same percentage rate; after 7 days of fasting the fatty groups still had slightly hypertrophied livers.

In the pair-feeding experiment on young rats, the daily food consumption increased during the 14-day period from 8.5 to 12.5 g. For the last few days fatties averaged 12 g heavier in the afternoon (a few hours after feeding) than in the morning. Half of both fatty and control groups were killed in the morning, half in the afternoon. Table 2 shows body weights and those other observations with large fluctuations in relation to time of feeding. According to these data, the restricted fatties gained a little less weight than the non-obese controls with the same food intake, and very much less weight than the control fatties fed ad libitum. Thus, the final stripped weights averaged 130 for fatties, and 138 for controls; initially the fatties were heavier, although the magnitude of this initial excess is exaggerated by the greater intestinal fill in fatties. The 2 fatties carried through with ad libitum feeding of the same diet (L15) arrived at 194 g (stripped weight 168) from a smaller starting weight. The large number of fatties of initial weight similar to that of the experimental group, and carried through the next 14 days on the stock diet regimen, reached 190 g.

In agreement with previous observations using chemical analytical procedures (2, 4), the serum lipid of fatties was above normal at 4 weeks of age (0.21 vs. 0.10%). In the restricted fatties, fasting greatly lowered the serum lipid (to 0.05%). This effect of an overnight fast in lowering serum lipid to the normal range is very dif-

TABLE 2

Paired feeding: body weight and serum composition in relation to time of feeding [1,2]

Time killed [3]	Body weight		Filled GI tract + supporting tissue	Serum	
	4-week [4]	6-week [4]		Lipid	Protein
	g	*g*	*g*	*%*	*%*
			Normal rats, fed ad libitum		
AM [3]	76 ± 2.5 [5]	155 ± 3.5	17.3 ± 2.5	0.16 ± 0.04	5.1 ± 0.3
PM [3]	73 ± 4.5	156 ± 5	17.4 ± 1.5	0.12 ± 0.02	5.1 ± 0.1
			Fatties, restricted		
AM	93 ± 4.5	147 ± 5	13.9 ± 0.5	0.05 ± 0.01	5.8 ± 0.2
PM	89 ± 10	151 ± 4	23.9 ± 1.5	0.39 ± 0.06	5.2 ± 0.5

[1] Three rats/group.
[2] Control data for 4-week-old rats fed ad libitum; 24 normals, body weight 74 (range ± 11, σ_M 2.3), lipid 0.10 (range ± 0.03); 12 fatties, body weight 95 g (range ± 11, σ_M 1.2), serum lipid 0.21 (range ± 0.05). Control data on 6-week-old male fatties fed ad libitum: 2 rats fed diet L15 went from an initial weight of 85 g to a final weight of 194 g; a further group of 19 on the stock diet regimen went from 91 g (range ± 16, σ_M 2.4) at 4 weeks to 190 g (range ± 46, σ_M 6.0) at 6 weeks.
[3] AM: before daily feeding of restricted fatties. PM: 3 to 4 hours after daily feeding. Fatties gained an average of 12 g during this interval; the fasted weight average for the 6 rats was 143 g, and the fed weight average for the 6 rats was estimated at 155 g.
[4] Initial age 27 to 30 days; duration of experiment 14 days.
[5] Mean ± average range.

ferent from the near absence of any effect of an overnight fast shown in table 1; whether the greater responsiveness of serum lipid shown by the rats of table 2 is attributable to their younger age, or to the continuing food restriction, is not known.

The contributions of various tissues to the gains made by the restricted rats and their controls are analyzed in table 3. Skeletal growth, as measured by the growth of a representative long bone, was greatly depressed by the food restriction. Growth of calf muscle was also greatly depressed. The restricted fatties, however, laid down over 3 times as much adipose tissue as the normals on the same food intake. Thus with a food restriction sufficient to impose a 40% reduction in weight gain, the fatty continued to increase its energy stores, at the expense of true growth.

TABLE 3

Paired feeding: increase in tissue weights of rats during 14-day experiment

df	Tibia length	Calf muscle [1]	Abdominal fat [1]
	cm³	*g*	*g*
		Normal rats, fed ad libitum	
28	12.4 ± 0.77 [2]	0.388 ± 0.022	1.35 ± 0.047
		Fatties, restricted	
16	8.3 ± 0.67	0.215 ± 0.017	4.12 ± 0.063

[1] See Experimental section for description of tissues weighed.
[2] Standard error of the difference between initial and final means.

The degree of adiposity and relationships among other tissues affecting bodily proportions are analyzed in figure 2. In the first graph we have plotted individual values of abdominal fat against the cube of tibia length. Normal proportions are represented by the line connecting the points for 4- and 6-week-old normal rats; any position on or near this line means normal fatness (6). The degree of obesity is measured by the distance above the line; since the scales are logarithmic, this distance corresponds to the ratio of fat pad weights of fatty and normal. According to this analysis, during the 14-day experimental period restricted fatties, despite the restriction, maintained their initial degree of obesity.

There is no inconsistency between the conclusion from table 3, that there was a greater than normal fat gain in the restricted fatties, and the conclusion from figure 2, that the initial degree of obesity was maintained. It is characteristic of the normal rat fed ad libitum that the percentage of fat increases continuously during growth (6); this is indicated by the very steep slope of the normal line in figure 2. The degree of obesity in an obese individual has to be evaluated against this background of increasing fat content in the normal rat; maintenance of constant obesity requires a continuously increasing excess in the amount of fat stored.

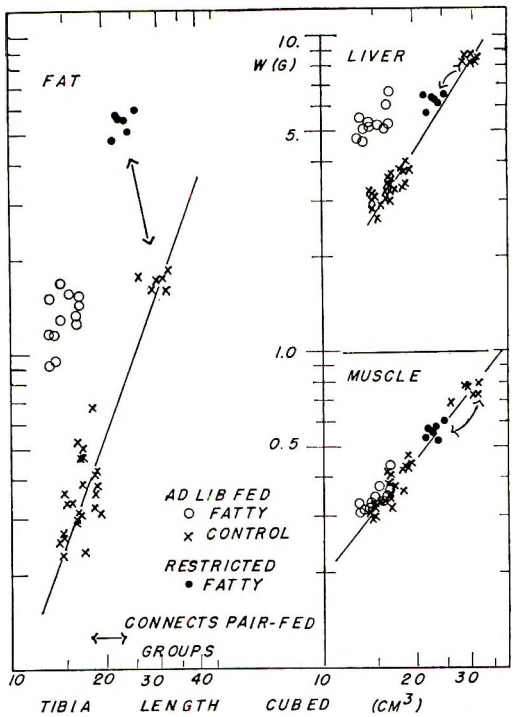

Fig. 2 Effect on adipose tissue, muscle and liver of pair-feeding fatties matched to normals. Rats fed ad libitum at the start of the experiment (4-week-old) are represented by open circles (fatties) and crosses (normals). The 6 fatties restricted for 2 weeks are represented by solid circles, and the 6 ad libitum-fed normals to whom the fatties were matched in food intake, by crosses. *Fat* indicates retroperitoneal + epididymal fat pads. *Muscle* indicates calf muscle mass (see Methods).

According to the second plot of figure 2, calf muscle was approximately normal in relation to tibia length in fatties. The effect of the food restriction was to limit the growth of both tissues proportionately.

The third plot in figure 2 shows the considerable liver hypertrophy in full-fed fatties. These heavy livers look normal; analysis of similar livers has shown that the fat content is above normal but not greatly so, far less than necessary to account for the doubling in weight. Restricting the food intake to normal restores liver weight to normal.

DISCUSSION

Fatties, young and old, respond adequately, and apparently normally, to total starvation. They live for a long period on their stored fat, and die when this is used up; normal rats die after a short time when their fat is used up, and live a suitably longer time if supplied with exogenous fat. In the early stages of fasting, the water consumption of fatties suggests that they are burning largely fat. In the early stages, while they still have fat stores, normal rats show a slow depletion of skeletal muscle, and fatties follow the same pattern.

However, if one meal per day is supplying a normal food intake, fat depots are abnormally spared; growth of non-fat tissues is inhibited to allow the continued growth of adipose tissue, and the rat remains obese despite persistent dieting. The maintenance of obesity at the expense of true growth is not restricted to the particular dietary fat level used in this experiment (somewhat over 20% fat by weight). A total of 36 fatties has been raised to adult age, and some until death (see life-span data (2)), on a restricted intake of a low fat diet. In some cases this was a commercial pelleted rat diet, and in others our lactation diet. They were all obviously obese, quite abnormal in their physique, and appeared to be smaller (shorter) than fatties usually are. The extent of the food restriction varied and was not well-defined, and no quantitative measurements were made with respect to degree of obesity and degree of retardation of skeletal growth. The experiments were not controlled, but are quite adequate to support the statement that one cannot render a fatty non-obese by a restricted intake of a low fat diet. The effect of calorically limited intakes of high fat, or high protein, or low carbohydrate diets remains to be explored.

The disproportion in growth observed in restricted fatties is just the reverse of that produced by growth hormone administered to rats fed a restricted caloric intake; under these conditions growth hormone diverts metabolites from fat synthesis to bone and muscle growth. We have no information yet as to growth hormone activity in fatties, or effects of exogenous growth hormone in calorically restricted fatties. However, several other observations are possibly pertinent. Even when fed ad libitum, fatties gradually fall behind their sibs in skeletal growth, and the difference reaches 15 to 20% (4) (table 1).

We find a continuing low incidence of really dwarf fatties, less than half the usual size for fatties, quite obese but with very small frames. In the early stages of carrying on the mutation, we relied fairly heavily upon the occasional fertile fatty male, and then we observed some deterioration in average size of the non-obese rats in this stock, as compared with the colony of fully normal rats of the same strain which we carry on by random mating. Since we have eliminated the use of fatty breeders in carrying on the fatty stock, and use only heterozygotes, this trend has stopped.

Impairment of skeletal growth, and a possible implication of growth hormone insufficiency, occur in association with obesity produced by surgical injury to the hypothalamus. Han and co-workers (7) have made careful measurements of skeletal growth in hypothalamically obese rats. Some growth depression was always present, there was a good correlation between degree of growth depression and degree of obesity, and obesity could be produced without excessive food intake, presumably at the expense of general somatic growth. Kennedy (8) had previously noted evidence of somatic growth impairment in young hypothalamically obese rats, with a correlation between degree of growth depression and degree of obesity. However, in Kennedy's operated rats there were also cases of growth depression without obesity, and obesity without growth depression. Kennedy concluded that his experimental lesion variably involved two different hypothalamic sites, one affecting obesity, the other one somatic growth. Han and coworkers reached the same conclusion, and in addition suggested that the growth depression is caused by a reduction in output of growth hormone. They cite recent evidence for a factor located in this general region of the hypothalamus, which controls the output of growth hormone by the pituitary; evidence for this new tropic hormone comes partly from observations on the effect of surgical injury (9), and more particularly from the ability of hypothalamic extracts to cause growth hormone release from the pituitary both in vivo (10) and in vitro (11).

If growth hormone activity is sufficiently depressed to affect skeletal growth, there should also be metabolic consequences of importance to the obesity problem. Thus the most sensitive response to exogenous growth hormone is fat mobilization in the fasting subject; this effect is produced by much less growth hormone than the level required to increase skeletal growth (12, 13).

We have cited the persistent diversion of a limited caloric intake to fat deposition, skeletal growth depression, and occasional severe stunting in our rats, as reasons for inquiring into the status of growth hormone in the fatty. The fatty does not show (spontaneous) lesions in the hypothalamus (footnote 3 in (6)). However, absence of visible lesions does not necessarily preclude a functional abnormality in the production of growth hormone or growth-hormone releasing factor.

In a previous report (4) we presented evidence that the liver of the fatty is characteristically enlarged at all ages, and that the enlargement is unchanged by large losses in body weight. The weight losses were taken to be evidence of spontaneously greatly reduced food intake, although no actual food intake measurements were made, and it is possible that some of these rats were losing appreciable quantities of protein in their urine. Adult fatties are frequently nephrotic. The data of table 2, for adult fatties in good condition subjected to forced starvation, show that the liver can be more responsive to fasting than we had thought. And the data of figure 2 show that in young rats, precise prevention of hyperphagia restores liver size to near normal. Whatever the state of responsiveness of the liver to food intake in older fatties, in young fatties the liver hypertrophy is largely, if not entirely, secondary to hyperphagia.

ACKNOWLEDGMENTS

Martha Thorne and Olive R. Hekkala provided valuable technical assistance.

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Reassessment of Some Amino Acid Requirements of Larvae of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) [1]

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ABSTRACT Dietary requirements of *Oryzaephilus surinamensis* (L.) for alanine, proline, cystine, and arginine were determined using a purified diet. Measurements of rate of survival and development showed that dietary components influenced amino acid requirements of the insect. Addition of alanine to the diet improved rate of survival or development, but only in the absence of nucleic acid. The indication that cystine is toxic to this insect under certain circumstances and that a concentration of 0.67% of arginine monohydrochloride in a diet containing nucleic acid is near the optimum was supported by the results. Requirements for lysine and methionine were apparently the same as had been reported previously. Alanine, arginine, and proline were among the bound amino acids of the nucleic acid used to supplement the basic diet, but cystine, lysine, and methionine were not. Until dietary nucleic acid can be replaced by substances of known composition, definitive dietary requirements of some amino acids cannot be determined.

Previous work with an artificial diet containing ribonucleic acid showed that larvae of *Oryzaephilus surinamensis* (L.) required small quantities (0.7 mg/g) both of alanine and of proline for optimal growth and development (1). It also determined the optimal quantities of dietary lysine (13.7 mg/g), methionine (5.6 mg/g), and arginine (6.7 mg/g) for this insect (2–4). Removal of cystine from this diet resulted only in a retardation of development (3). Recently, addition of yeast ribonucleic acid to a purified diet developed originally for *Tribolium confusum* Duval [2] improved that diet, so that *O. surinamensis* developed more rapidly than with the artificial diet used previously (5).

Dietary requirements of insects are influenced by changes in dietary constituents and are correlated with metabolic changes arising directly from altering dietary components (6, 7). To determine whether amino acid requirements reported earlier for *O. surinamensis* were still valid when a modified Medici diet was used, alanine or proline, or both, were added to the diet, cystine was deleted, and arginine was included at a concentration lower than in the original Medici diet.[3]

The present paper reports on the effects of such dietary alterations on survival and rate of development of larvae of *O. surinamensis*.

EXPERIMENTAL

The control diet (table 1) was based on a synthetic medium used for *T. confusum*.[4] Eleven other diets were prepared by single or multiple additions of alanine (0.7 mg/g), proline (0.7 mg/g), and yeast ribonucleic acid (5.0 mg/g), by reduction of arginine (to 6.7 mg/g), or by omission of cystine.

One hundred newly hatched larvae of *O. surinamensis* were reared individually with each diet at 32 ± 2° and 75 ± 5% relative humidity, as in previous investigations (1). They were inspected every 24 hours and survival and development were recorded. These larvae were obtained from eggs laid by adult beetles during a 24-hour period. Adults were kept in an egg-farm of vitamin-enriched flour, supplemented with 3% of wheat germ, at the same temperature and humidity. Fresh supplies of adult

Received for publication April 4, 1966.

[1] Contribution no. 248, Canada Department of Agriculture Research Station, Saskatoon, Saskatchewan.

[2] Medici, J. C. 1964 Nutritional studies including quantitative amino acid and mineral requirements with the flour beetle, *Tribolium confusum* Duval. Ph.D. Thesis. Rutgers — The State University, New Brunswick, New Jersey.

[3] See footnote 2.

[4] See footnote 2.

TABLE 1

Composition of the control diet used to determine dietary amino acid requirements for Oryzaephilus surinamensis (L.)

Amino acids [1]	mg/g	Other components [1]	mg/g
L-Arginine (free base)	9.0	Cornstarch [2]	725.0
L-Cystine	2.0	Cholesterol, PH	3.7
L-Glutamic acid	30.0	Salt mixture (M-D no. 185) [3]	35.0
Glycine (free of ammonia)	53.0	Folic acid	0.2
L-Histidine (free base)	6.0	Choline chloride	1.5
L-Isoleucine	12.0	*i*-Inositol	1.5
L-Leucine (methionine free)	14.0	Thiamine · HCl, USP	0.15
L-Lysine monohydrochloride	13.0	Riboflavin	0.15
L-Methionine	6.0	Niacin	0.15
L-Phenylalanine	5.0	Pyridoxine · HCl, PH	0.15
L-Threonine (allo-free)	9.0	Ca D-pantothenate	0.15
L-Tryptophan	2.0	*p*-Aminobenzoic acid, NF	0.15
L-Tyrosine	10.0	Biotin (free acid) [4]	3.0×10^{-7}
L-Valine	11.0	Vitamin B_{12} [4]	3.6×10^{-7}
			ml/g
		Corn oil [2]	0.01

[1] Mann Assayed grade, Mann Research Laboratories, Inc., New York, except where otherwise noted.
[2] Best Foods Division of The Canada Starch Company, Ltd., Montreal, Quebec.
[3] McCollum, E. V., and M. Davis 1914 Observations on the isolation of the substance in butterfat which exerts a stimulating influence on growth. J. Biol. Chem., *19*: 245.
[4] Nutritional Biochemicals Corporation, Cleveland.

beetles, obtained from stock rearing, were added to this farm at approximately 6-week intervals. The larvae used in these investigations hatched from eggs oviposited over a one-month period. Experience at this laboratory has indicated little variation in survival or development time with any specific diet from one year to the next, at this temperature and humidity. During a 10-year period (1956–1965), larvae of *O. surinamensis*, fed an artificial diet containing casein as the protein source, had an average survival to pupation of 89% (range, 78% – 100%) and an average rate of development of 517 hours (range, 467 ± 4 [5] hours to 595 ± 14 hours. The average percentage of pupae becoming adults was 87% (range, 85% to 100%) and the average development time to emergence was 611 hours (range, 578 ± 5 hours to 657 ± 22 hours). No apparent lack of vigor was observed during this period. Tests are run periodically with this casein diet to determine any change in the insect population. When extreme variations occur between replicates fed the same diet or when results differ greatly from the expected, the tests are repeatd several times to determine the real effect of such diets. Under such conditions, comparison of tests can be made, although they are not all initiated on the same day nor from the same batch of eggs.

The chi-square test (8) was used to determine differences in survival and Duncan's multiple range test (9) to determine differences in rate of development. Differences at the 5% level of confidence were considered significant. To assess the efficiency of the various diets for the two life-history stages observed, the concept of the environmental index (10) was applied to the data. The relative environmental index for each development stage is obtained by dividing the percentage survival of that stage by the average development time. The absolute environmental index for each stage is derived by converting the highest value of the relative environmental index to unity and by adjusting the other values in the same way. The cumulative relative environmental index for any diet is the product of the absolute values for each growth stage considered. To obtain the cumulative absolute environmental index for each of several diets being compared, the same conversion as above is applied.

[5] Average ± SE of mean.

RESULTS

O. surinamensis survived better or developed earlier, or both, when fed modified diets (table 2) than when fed the basic diet. More larvae survived to pupation with all modified diets, except with those supplemented only with alanine or with alanine and proline, than with the basic diet. With all modified diets, except that to which only proline was added, they developed to the pupal stage earlier than with the basic diet. More adults emerged in populations fed modified diets than in those fed the control, except when the diet lacked cystine and was supplemented only with nucleic acid, when it was supplemented with alanine and proline only, or when it contained alanine, proline, cystine and nucleic acid (table 2). With all modified diets, the adults emerged earlier than with the control diet, except when proline only was added.

O. surinamensis developed at the same rate with the basic diet and with the basic diet to which only proline was added. It developed faster than with the basic diet ($P < 0.05$) with diets supplemented with alanine only; with alanine and proline only; with alanine, proline, 6.7 mg/g of arginine, and nucleic acid; or with alanine, proline, and nucleic acid in the absence of cystine. It developed most rapidly, however, with the six other diets (table 2).

The absolute environmental index (table 3) showed that larvae survived and developed best with a diet to which only nucleic acid had been added. However, the most efficient production of adults from pupae occurred with the basic diet. When both stages were considered, the basic diet was intermediate in performance and that to which only nucleic acid was added was the most efficient (table 3).

If diets containing nucleic acid are disregarded, the most efficient diet for larval development was one to which only alanine had been added. It was followed closely by diets containing alanine and proline, proline alone, and by the control diet. Similarly, of the diets lacking nucleic acid, the control diet was the most efficient for production of adults from pupae, taking into consideration both numbers of adults and time spent in the pupal stage. It was followed in order by those diets supplemented with proline alone, alanine and proline, or alanine alone. For the combined stages, the nucleic acid-deficient diet supplemented with alanine only was the most efficient, followed by the control diet, by the diet supplemented with alanine and proline, and by that supplemented with proline alone.

In the presence of nucleic acid and in the absence of cystine, fewer larvae survived to pupation ($0.05 > P > 0.02$) and fewer adults emerged from the pupae ($P < 0.01$) than when cystine was included in the diet (table 2). However, when cystine was omitted from a diet con-

TABLE 2

Effects on survival and rate of development of 100 individuals of Oryzaephilus surinamensis *(L.) reared at 32 ± 2° and 75 ± 5% relative humidity with the basic diet and with various modified diets*

Components		No. pupating	Avg time [1]	No. emerging	Avg time [1]
Added	Deleted				
			hours		*hours*
None	none	66	680 ± 25 [2,a]	52	756 ± 27 [a]
L-Alanine	none	74	572 ± 16 [b,c]	67 [3]	686 ± 16 [b]
L-Proline	none	78 [3]	657 ± 16 [a]	71 [3]	771 ± 14 [a]
RNA	none	86 [3]	467 ± 6 [e]	78 [3]	587 ± 6 [e]
RNA	L-cystine	79 [3]	476 ± 6 [d,e]	60	607 ± 8 [d,e]
RNA	L-arginine (2.3 mg/g)	80 [3]	499 ± 8 [d,e]	65 [3]	623 ± 8 [d,e]
L-Alanine, RNA	none	87 [3]	476 ± 5 [d,e]	63 [3]	586 ± 7 [e]
L-Proline, RNA	none	82 [3]	497 ± 10 [d,e]	65 [3]	608 ± 8 [d,e]
L-Alanine, L-proline	none	75	592 ± 21 [b]	58	689 ± 21 [b]
L-Alanine, L-proline, RNA	none	84 [3]	507 ± 9 [d]	61	640 ± 12 [c,d]
L-Alanine, L-proline, RNA	L-cystine	82 [3]	546 ± 7 [c]	72 [3]	670 ± 8 [b,c]
L-Alanine, L-proline, RNA	L-arginine (2.3 mg/g)	77 [3]	547 ± 10 [c]	66 [3]	679 ± 9 [b]

[1] Average times not followed by the same letter are significantly different from one another at the 5% level of confidence.

[2] SE.

[3] Significantly greater than that obtained with basic diet at 5% level of confidence.

TABLE 3

Values of the absolute environmental index obtained for Oryzaephilus surinamensis *(L.) reared at 32 ± 2° and 75 ± 5% relative humidity on the basic diet and on various modified diets*

Components		Absolute environmental index		
Added	Deleted	Larval development	Pupal development	Cumulative
None	none	0.527(12)[1]	1.000(1)	0.723(7)
L-Alanine	none	0.703(9)	0.759(4)	0.742(6)
L-Proline	none	0.645(11)	0.768(2)	0.679(10)
RNA	none	1.000(1)	0.729(5)	1.000(1)
RNA	L-cystine	0.902(3)	0.558(11)	0.690(9)
RNA	L-arginine (2.3 mg/g)	0.871(6)	0.628(9)	0.750(5)
L-Alanine, RNA	none	0.993(2)	0.629(8)	0.857(2)
L-Proline, RNA	none	0.896(5)	0.685(6)	0.842(3)
L-Alanine, L-proline	none	0.688(10)	0.764(3)	0.721(8)
L-Alanine, L-proline, RNA	none	0.900(4)	0.528(12)	0.652(12)
L-Alanine, L-proline, RNA	L-cystine	0.816(7)	0.683(7)	0.764(4)
L-Alanine, L-proline, RNA	L-arginine (2.3 mg/g)	0.765(8)	0.627(10)	0.659(11)

[1] Numbers in parentheses indicate rank attained by various stages with different diets.

taining nucleic acid, alanine, and proline, the larvae survived to pupation as well as with the same diet to which cystine was added ($0.70 > P > 0.50$), but more adults emerged with the cystine-deficient diet than with that containing cystine ($0.05 > P > 0.02$).

Larval survival to pupation with a diet supplemented with nucleic acid only, was unaffected (table 2) by reducing the arginine content from 9.0 mg to 6.7 mg/g ($0.10 > P > 0.05$), but adult emergence was decreased ($P < 0.01$). In contrast, with a diet containing nucleic acid, alanine, and proline, survival of *O. surinamensis* both to the pupal ($0.10 > P > 0.05$) and to the adult stage ($0.50 > P > 0.30$) was unaffected by reducing the concentration of arginine. Conversely, when the concentration of arginine in diets containing nucleic acid was reduced to 6.7 mg/g, the rate of development of *O. surinamensis* was decreased, whether alanine and proline were included or not (table 2). Nevertheless, this retardation of development was more apparent when alanine and proline were included in a diet supplemented with nucleic acid than when they were not.

DISCUSSION

These results show that the dietary requirements of *O. surinamensis* for amino acids were influenced by the components of the diet. Earlier investigations (1) showed that in the presence of ribonucleic acid small amounts of alanine and proline were necessary for normal survival and development of *O. surinamensis*. In contrast, present results show that addition of alanine, in the absence of nucleic acid, tends to improve the basic diet (table 3); no such beneficial effects were obtained in the presence of nucleic acid.

Differences between the results obtained in the 2 investigations may have arisen from differences in the composition of the nucleic acid used or from differences in the relative concentrations of the other components. Differences, as measured by performance of larvae of *O. surinamensis*,[6] do arise when commercial yeast nucleic acid from other manufacturers is used in the diet.

Paper chromatography of an aqueous extract of the ribonucleic acid used in this investigation failed to reveal the presence of any free amino acids. However, preliminary paper chromatography of a hydrolysate of this yeast ribonucleic acid obtained with 3 N HCl at 120° for 5 hours showed that alanine, arginine, aspartic acid, glutamic acid, glycine, serine, tyrosine, and possibly histidine and proline were present in bound form. Gas-liquid chromatography of the same hydrolysate corroborated these observations, although the technique used did not permit identification of arginine, cystine, or histidine. Lysine, methionine, and threonine were not present according to the gas-liquid chromatographic analysis of the hydrolysate.

Addition of this ribonucleic acid to the diet supplied, to some extent at least, the

[6] Unpublished data, G. R. F. Davis, 1965.

alanine, aspartic acid, proline, and serine not included in the basic diet (table 1). It also increased the concentration of other amino acids over that indicated in the table. This addition, then, precludes determination of definitive requirements of *O. surinamensis* for alanine, arginine, and proline. Nevertheless, determination of differences arising from variations in the dietary components of two artificial diets containing the ribonucleic acid from the same source was possible.

Because cystine was not demonstrated as a bound amino acid in the ribonucleic acid used, the results of this investigation probably reflect the effect of dietary cystine on growth and development of this organism. Previously, it was shown that the only adverse effect of omitting cystine from a diet for *O. surinamensis,* in which alanine, proline, and nucleic acid were included, was a decrease in the rate of development (3). In agreement with these results, present results (table 2) indicate that deletion of cystine from a diet containing alanine, proline, and nucleic acid, does retard the rate of development. Also, in this investigation, deletion of cystine from such a diet resulted in better survival to the adult stage than that obtained with the control diet or with the diet containing alanine, proline, nucleic acid, and cystine. In contrast, deletion of cystine from a diet supplemented only with nucleic acid had no effect on development time, but reduced survival to the adult stage to a level equal to that obtained with the control diet (table 2). These results, therefore, lend support to the observation that cystine is toxic to *O. surinamensis* (3); however, they also show that the manifestation of this toxicity is dependent upon dietary components. They also indicate that some interaction may occur among alanine, cystine, proline, and nucleic acid affecting the ability of *O. surinamensis* to survive to the adult stage.

With diets containing 9.0 mg of arginine/g, fewer adults emerged when alanine, proline, and nucleic acid were included than when only nucleic acid was added to the basic diet, but their rate of development was unaffected. With similar diets containing 6.7 mg of arginine/g, more adults emerged when alanine and proline were included with nucleic acid than when they were omitted, but they developed slower. Arginine monohydrochloride was used in the previous investigation (4) and arginine free base in the present work. However, the concentration of 9.0 mg of arginine free base/g corresponds closely, on an equimolecular basis, with the amount of arginine monohydrochloride (0.67%) shown to be optimum in that investigation. Similarly, the concentration of 6.7 mg of arginine free base/g corresponds to a slightly lower concentration of arginine monohydrochloride (0.55%) than the optimum demonstrated earlier. A decrease in the arginine concentration from the optimum in a diet containing alanine, proline, and nucleic acid delayed development in both investigations, but did not affect survival. The present results (table 2) support the view that a concentration of 0.67% of arginine monohydrochloride is close to the optimum for this insect, when alanine and proline are present in a diet supplemented with nucleic acid. The presence of bound arginine in the nucleic acid, however, necessitates a redetermination of optimal arginine requirements, when dietary nucleic acid can be replaced by substances of known composition.

The diet used in the present investigation contained lysine and methionine in concentrations considered nearly optimal (2, 3). Neither amino acid was observed, free or bound, in the nucleic acid added to the basic diet. Therefore, the results were a reflection of the dietary requirements of *O. surinamensis* for these amino acids. The requirements for lysine and methionine with the present diet must have been the same as reported previously. Otherwise, survival and development with a diet containing alanine, proline, nucleic acid, and these amino acids would not have been equal to or better than those obtained with the previous diet (2, 3). Apparently, the dietary changes of the present investigation did not modify the requirements of *O. surinamensis* for these amino acids as they did for alanine, proline, and probably cystine.

ACKNOWLEDGMENTS

The technical assistance of F. T. Eves is gratefully acknowledged. The gas-liquid

chromatography of the amino acid hydrolysate was performed by Dr. J. G. Saha of this Research Station.

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Effects of Glucose Supplementation of High Lipid Diets Based on Free Fatty Acids for the Growing Chicken [1,2]

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ABSTRACT Three experiments were conducted with chicks to evaluate the effects of glucose supplementation in diets high in soybean oil fatty acids (SOFA). Semi-purified diets based on isolated soybean protein were used. The high fatty acid diet supplied 68% of total metabolizable calories as SOFA and the remaining 32% as protein. Supplemental glucose replaced an equicaloric amount of SOFA. Cellulose was added to maintain a mealy texture. The addition of glucose to the high fatty acid diet increased both food intake and growth. Over the range 1.5% to 18% glucose calories, the voluntary food intake provided nearly a constant amount of SOFA approximating 42 g/chick for the first 2 weeks. In contrast, the lipid intake of chicks fed the diets high in soybean oil was almost 60 g/chick. This suggests that food intake is regulated to avoid exceeding a limit on SOFA intake. The level of glucose in the blood of chicks fed the high SOFA diet was subnormal during the absorptive condition but increased to normal post-absorptive values after a fasting of 18 hours. Chicks fed diets high in SOFA were very lean, whereas those recieving the high soybean oil diet deposited as much fat as the reference group fed a high carbohydrate diet. The fatty acid composition of depot fat from chicks receiving the high lipid diets was highly unsaturated and resembled closely that of soybean oil.

We have reported that feeding diets in which the non-protein calories are supplied as soybean oil fatty acids (SOFA) greatly retards the growth of chicks and induces hypoglycemia and severe ketonemia (1). Supplementation of this diet with glucose to supply 6% of the calories markedly increases rate of growth, and produces normal levels of blood glucose and ketone bodies. We also observed that the improved growth produced by supplementation with glucose was associated with enhanced food consumption and increased fatty acid intake.

The question arose as to whether the progressive substitution of glucose for SOFA in the high fatty acid diet would produce further increases in rate of growth, food consumption and fatty acid intake. The results of our study reported herein indicate that the young chick has a limited capacity to utilize dietary fatty acids, and that this limitation is unrelated to its requirement for an exogenous source of carbohydrate. Data on blood glucose, carcass analysis and fatty acid composition of depot fat of chicks fed high lipid or high carbohydrate diets are also presented.

EXPERIMENTAL

Description of animals, housing, analytical techniques and statistical analysis are summarized in our previous communication (1). As before, each diet was fed to duplicate lots of 10 one-day-old, male chicks. Food and water were given ad libitum. Individual body weights and group food consumption were recorded at weekly intervals.

For the determination of body composition, the chicks were fasted for 16 to 18 hours and then killed by chloroform inhalation. Each sample consisted of a composite of the 3 chicks closest to the average weight for that particular lot. The carcasses were frozen, chopped, ground in a meat grinder and finally homogenized in a Hobart bowl mixer. A representative portion of this homogenate was dried in shallow pans in a forced-draft oven at 50° to

Received for publication August 15, 1966.
[1] Supported in part by a Rockefeller Foundation fellowship awarded to the senior author.
[2] Reported in part at the 1965 meeting of the American Institute of Nutrition. Hill, F. W., and S. Brambila 1965 Properties of high lipid diets based on free fatty acids. Federation Proc., 24: 501 (abstract).
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55° for 24 hours. The dry material was ground finely in a Wiley mill and mixed to ensure uniformity. Residual moisture was determined in a vacuum oven at 55° to 60°; total nitrogen was determined by macro-Kjeldahl; carcass lipids were determined by extraction with anhydrous ethyl ether in a Goldfisch apparatus.

Composite samples of abdominal fat of 5 chicks per group were extracted with chloroform-methanol (2), using water-partitioning to remove non-lipid contaminants. The lipids present in the chloroform layer were hydrolyzed (3), and the resulting fatty acids converted to the corresponding methyl esters with methanol-BF_3 (4). The fatty acid composition of depot fat was determined by gas-liquid chromatography of the methyl esters (1).

Diets. The composition of the high carbohydrate reference diet is shown in table 1. It is similar to that used in our previous work (1) and supplied in percentage of metabolizable calories: glucose, 45; protein, 32; and soybean oil (SO), 23. It was modified from our previous diet to supply increased levels of most vitamins, approximating 5 to 10 times the requirements as estimated by NRC (6); vitamins A and D, and choline, were supplied at levels approximately equivalent to twice the estimated requirements. The mineral mixture was also modified to improve the balance of Na, K and Cl (7).

TABLE 1

Composition of the high carbohydrate reference diet

	% dry matter
Glucose [1]	48.85
Isolated soybean protein [2]	31.00
Soybean oil	10.00
DL-Methionine	0.70
Glycine	1.00
Mineral mixture [3]	7.41
Vitamin mixture [4]	0.72
Chromic oxide	0.30
Antioxidants [5]	0.02

[1] Anhydrous Cerelose, Corn Products Company, Argo, Illinois.
[2] C-1 Assay protein, Archer-Daniels-Midland Company, Minneapolis.
[3] Mineral mixture supplied in mg/100 g diet: $CaHPO_4 \cdot 2H_2O$, 3600; $CaCO_3$, 1000; Na_2HPO_4, 600; KCl, 680; NaCl, 100; $NaHCO_3$, 580; $KHCO_3$, 400; $MgSO_4$, 350; $MnSO_4 \cdot H_2O$, 25; ferric citrate, 50; $ZnCO_3$, 20; $CuSO_4$, 2; KIO_3, 1; Na_2MoO_4, 0.8; cobaltous acetate, 0.18; and Na_2SeO_3, 0.022.
[4] Vitamin mixture supplied in mg or units/100 g diet: niacin, 15; Ca pantothenate, 10; pyridoxine · HCl, 2; riboflavin, 1.5; thiamine · HCl, 1; folacin, 0.3; menadione, 0.3; biotin, 0.1; cyanocobalamin, 0.01; choline chloride, 200; vitamin A palmitate, 500 IU; vitamin D_3, 50 ICU; and *d*-α-tocopheryl acetate, 10 IU.

For the preparation of the high lipid diets, both glucose (48.85 g) and SO (10 g) were replaced equicalorically with the lipid under study (29.2 g SO; 30.70 g SOFA[4]). The estimated metabolizable energy (ME) content of SOFA was increased from 8.50 kcal/g used in our previous study to 8.80 kcal/g on the basis of a determined absorbability of 93% rather than 90% (1). Supplementation of these diets with glucose was made by replacing an equicaloric amount of lipid. Cellulose [5] was added to the high lipid diets to produce a mealy texture. For the series of SOFA diets the level of added cellulose decreased as that of glucose increased, so that the caloric density of most diets, expressed as kilocalories ME per gram, remained constant.

RESULTS

The effects of the progressive substitution of glucose for SOFA on growth, energy consumption and fatty acid intake for the 2-week-old chick are shown in table 2. As observed before (1), diets in which all the non-protein calories were supplied by SOFA greatly retarded rate of growth and reduced food intake. Supplementation of the high fatty acid diet with as little as 1.5% glucose calories significantly increased both rate of growth and food consumption. Further supplementation with glucose in a step-by-step fashion, progressively improved growth and food intake, but it was necessary to reach a very high proportion of calories from glucose to obtain growth rates which approached those of the chicks fed the high neutral fat or the high carbohydrate reference diet. In confirmation and extension of our previous observation (1), the cumulative intake of SOFA over a 2-week period was increased by the addition of glucose to the high fatty acid diet. Over the range of 1.5% to 18% glucose calories, the voluntary intake of all SOFA-base diets resulted in a relatively constant intake of 41 to 44 g SOFA/chick over the 14-day period. At glucose levels above 18% of calories, the fatty acid intake diminished progres-

[4] Wecoline S, kindly supplied by Drew Chemical Company, Inc., New York.
[5] Solka Floc BW-200, Brown Company, Boston.

TABLE 2

Growth and food intake of chicks fed diets high in fatty acids with increasing amounts of glucose

Treatment [1,2]	Experiment 5			Experiment 8		
	Avg wt, 2 weeks	ME intake (calculated)	Lipid intake	Avg wt, 2 weeks	ME intake (calculated)	Lipid intake
	g	*kcal/chick*	*g/chick*	*g*	*kcal/chick*	*g/chick*
Glucose (0) + SOFA (68)	120 [a 3]	433	33 [a]	124 [a]	478	36 [a]
Glucose (1.5) + SOFA (66.5)	—	—	—	159 [b]	549	42 [b]
Glucose (3) + SOFA (65)	149 [b]	547	41 [bc]	163 [bc]	590	44 [b]
Glucose (6) + SOFA (62)	153 [b]	576	41 [bc]	168 [bcd]	599	42 [b]
Glucose (12) + SOFA (56)	167 [c]	659	41 [bc]	177 [bcd]	638	41 [b]
Glucose (15) + SOFA (53)	174 [cde]	691	42 [b]	—	—	—
Glucose (18) + SOFA (50)	169 [cd]	684	39 [cd]	195 [cdef]	726	42 [b]
Glucose (24) + SOFA (44)	179 [de]	752	38 [d]	183 [bcde]	716	36 [a]
Glucose (30) + SOFA (38)	183 [e]	724	32 [a]	199 [def]	757	33 [a]
Glucose (36) + SOFA (32)	—	—	—	202 [def]	776	28 [c]
Glucose (45) + SOFA (23)	—	—	—	214 [efg]	811	21 [d]
High soybean oil (68)	206 [f]	790	58 [e]	220 [fg]	804	59 [e]
High glucose reference diet	221 [g]	801	20 [f]	242 [g]	850	22 [d]

[1] Parenthetic figures are % metabolizable calories supplied by ingredient; remainder, 32%, was from protein.
[2] Soybean oil fatty acids.
[3] Values are averages of duplicate groups. Values with differing superscript are significantly different ($P < 0.05$).

sively. The total amount of neutral fat eaten by the chicks fed the high SO diet is in sharp contrast with the much lower values for the fatty acid diets with or without carbohydrate supplementation.

Equicaloric substitution of glycerol for glucose at the 12% level, did not appreciably modify body weight at 2 weeks (171 g), energy consumption (635 kcal ME/chick) or cumulative fatty acid intake (41 g/chick). This is further evidence of the nutritional equivalency for the chick of glucose and glycerol calories under these conditions (1, 8).

Apparent lipid absorbability was close to 90% for the free fatty acids and 95% for SO.

Comparative data on blood glucose of chicks fed high lipid and high carbohydrate diets are shown in table 3. In confirmation of our earlier report (1), the high SOFA diet induced significant hypoglycemia which was prevented by the supplementation with 6% glucose calories. However, when blood glucose was measured under fasting conditions (18 hours, post-feeding), the level of glucose in the blood of chicks fed high SOFA diets was essentially the same as that observed for the high carbohydrate reference group. It was also found that the level of circulating glucose increased upon fasting for the chicks receiving the high fatty acid diet, whereas it decreased consistently for all

TABLE 3

Effect of moderate fasting (18 hours) on the level of blood glucose of chicks fed high lipid diets

Treatment [1]	Blood glucose			
	Exp. 8 [2]		Exp. 8A [3]	
	Fed	Fasted	Fed	Fasted
	mg/100 ml		*mg/100 ml*	
High glucose reference diet	223 [a 4]	179 [k]	222 [a]	204 [k]
High soybean oil (68)	184 [bc]	177 [k]	214 [ab]	188 [k]
High SOFA [5] (68)	169 [c]	185 [k]	175 [c]	198 [k]
Glucose (6) + SOFA (62)	205 [ab]	182 [k]	—	—

[1] Parenthetic figures are % metabolizable calories supplied by ingredient; remainder, 32%, was from protein.
[2] Five-week-old chicks.
[3] Four-week-old chicks.
[4] Values are averages of 10 chicks. Values with differing superscript are significantly different ($P < 0.05$).
[5] Soybean oil fatty acids.

TABLE 4

Gross body composition of chicks fed high lipid diets. Data on dry matter basis

Treatment [1]	Experiment 8 [2]			Experiment 8A [3]		
	Avg wt	Protein [4]	Fat	Avg wt	Protein [4]	Fat
	g	%	%	g	%	%
High glucose reference diet	794	59.0 [ab 5]	27.2 [a]	580	58.9 [a]	29.1 [a]
High soybean oil (68)	695	56.0 [a]	32.2 [b]	500	56.0 [a]	30.1 [a]
High SOFA [6] (68)	368	62.6 [b]	21.5 [c]	287	65.4 [b]	19.7 [b]
Glucose (6) + SOFA (62)	465	62.9 [b]	22.5 [c]	—	—	—
Glucose (30) + SOFA (38)	482	63.1 [b]	23.0 [ac]	—	—	—

[1] Parenthetic figures are % metabolizable calories supplied by ingredient; remainder, 32%, was from protein.
[2] Five-week-old chicks.
[3] Four-week-old chicks.
[4] N × 6.25.
[5] Values are the average of 2 composite samples of 3 chicks each. Values with differing superscript are significantly different ($P < 0.05$).
[6] Soybean oil fatty acids.

TABLE 5

Fatty acid composition of abdominal fat of 4-week-old chicks fed high lipid diets, compared with composition of soybean oil (SO)

Fatty acid	Diet [1]			Soybean oil composition
	Glucose (45) + SO (23) [2]	SO (68) [3]	SOFA [4] (68)	
	%	%	%	%
14:0	0.5	—	0.2	—
16:0	19.2	11.8	9.2	10.6
17:0 (?)	—	—	0.6	—
18:0	6.3	4.6	5.0	3.5
16:1	2.9	0.2	1.3	—
18:1	29.3	25.9	25.3	24.0
18:2	37.3	53.3	53.9	55.0
18:3	4.5	4.2	4.5	6.9

[1] Parenthetic figures are % metabolizable calories supplied by ingredient; remainder, 32%, was from protein.
[2] High carbohydrate reference diet.
[3] Soybean oil.
[4] Soybean oil fatty acids.

other treatments. These observations were confirmed in a second experiment (table 2, exp. 8A).

Data on body composition of chicks fed high lipid versus high carbohydrate diets are given in table 4. On a percentage basis, the high SO diet induced the deposition of more fat and less protein than any other treatment. In contrast, the chicks fed the high SOFA diet were extremely lean, and the supplementation with as much as 30% glucose calories failed to increase fat deposition significantly.

The fatty acid composition of abdominal fat of chicks fed high lipid and high carbohydrate diets is shown in table 5. As compared with the high carbohydrate reference group, the abdominal fat of chicks receiving the high lipid diets contained less saturated and more polyunsaturated fatty acids and resembled closely the composition of SO. A peculiarity of the fat from the chicks fed the high SOFA diet was the presence of a fatty acid which appeared in the chromatogram between $C_{16:1}$ and $C_{18:0}$ and which was tentatively identified as $C_{17:0}$. No similar peak was recorded for the mixture of SOFA, nor for the abdominal fat of chicks receiving the high neutral fat or the high carbohydrate diet.

DISCUSSION

The severe growth retardation exhibited by chicks fed diets in which almost all the non-protein calories are supplied by SOFA, and the severe ketonemia, are clearly due in part to carbohydrate deficiency. However, the failure of supplemental glucose to support normal growth rate, even though only small amounts are needed to correct the ketonemia, indicate further

specific growth-limiting properties of the mixture of fatty acids unrelated to carbohydrate deficiency.

The data shown in table 1 indicate that the progressive substitution of glucose calories for SOFA calories increased the level of voluntary food consumption up to the point where it reached a maximal cumulative intake of SOFA of approximately 42 g/chick for the first 2 weeks of life. The sharp increase in fatty acid intake from approximately 35 g for the chicks fed the all-SOFA diet to 42 g for those receiving as little as 1.5% glucose calories, may be of special significance, and suggests that this level of glucose was enough to satisfy a minimal requirement for carbohydrate.

The evidence presented suggests that SOFA limits the level of voluntary food intake. In the first 2 weeks of life, this limit approximates 42 g of SOFA per chick. The high carbohydrate and high SO diets support maximal growth with a calorie intake of approximately 800 kcal ME in this period. If SOFA intake is a primary factor limiting food intake, it would be expected that energy consumption would approach normal (i.e., 800 kcal) when the level of SOFA is such that the limit (40 g) is not exceeded when this level of energy consumption is approached. The data in table 2 show that ME intake increases as the glucose level is increased above 18% of calories, which is the point at which energy rather than fatty acid intake should begin to exert the limiting effect.

The very large difference between the total amount of lipid absorbed by the chicks fed the high SO diet and the SOFA diets suggests that a saturation point of the absorptive capacity for free fatty acids was reached. If it is assumed that the absorption of the mixture of SOFA involves their esterification into triglycerides at the intestinal mucosa (9), then the glycerol phosphate-phosphatidic acid pathway must be the main if not the only operative scheme for their esterification. In contrast, the direct monoglyceride pathway may be of major significance for the neutral fat diets. If the rate of esterification by the glycerophosphate pathway is considerably less than that of the monoglyceride path, then the limited capacity of the chick to utilize dietary fatty acids might be explained. The chicks fed the high SOFA diet without supplemental glucose might be even more limited in ability to utilize fatty acids on account of a superimposed carbohydrate deficiency which would restrict the amount of endogenous glucose which could be diverted to the synthesis of glycerol. Of relevance to this discussion is the fact that the adipose tissue (abdominal pad) of chicks fed the high fatty acid diet was composed mainly of triglycerides with only a barely detectable amount of free fatty acids (by thin-layer chromatography).

The chicks receiving the high SOFA diet showed what may be termed an "alimentary hypoglycemia" (table 2). In other words, the level of circulating glucose was subnormal during the absorptive condition and then rose to normal post-absorptive levels after a fast of 18 hours. This is in sharp contrast with the high carbohydrate reference group in which a similar fasting caused a decrease of the blood glucose level in agreement with previous reports (10, 11). This "reverse" effect of fasting on glycemia may be interpreted as the result of increased glycerol synthesis from endogenous glucose to satisfy the demand for the esterification of the dietary free fatty acids into glycerides.

The leanness of the chicks fed diets containing SOFA with or without supplemental glucose (table 3) may be the result of reduced food intake rather than of a specific property of the fatty acids themselves. In support of this view, it has been shown that deliberate restriction of food consumption to levels below 50% of the ad libitum intake did not completely stop early chick growth but caused a net loss of body fat after 2 weeks (12). The higher proportion of body fat in the chicks fed the high neutral fat as compared with those fed the high carbohydrate reference diet may be the result of more efficient utilization of energy in the high SO diet (13).

The observation that the fatty acid composition of depot fat closely resembled that of the dietary lipid (table 5) is not unexpected (14–16). However, the concept that the polyunsaturated fatty acids are usually deposited at the expense of oleic acid without altering the total proportion

of saturated fatty acids (17) is not supported by the data under consideration. With a fat-free diet, the depot fat of the growing chicken has been reported to contain 24% palmitic and 6% stearic acid (15). Table 5 shows that the relative proportion of saturated fatty acids was 26% for the chicks fed the high carbohydrate reference diet, but only 15% for those receiving the high lipid diets. This is perhaps to be recognized as a specific effect of the extreme nutritional condition in which all the non-protein calories were supplied by a highly unsaturated lipid. The significance of the extra fatty acid ($C_{17:0}$?) which could be detected only in the abdominal fat of chicks fed the high SOFA diet is uncertain and requires further work.

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Influence of Dietary Fat Level on the Enzymatic and Lipogenic Adaptations in Adipose Tissue of Meal-fed Rats [1]

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ABSTRACT The effect of increasing dietary fat levels on the response to meal-eating (restriction of food ingestion to a single daily 2-hour meal) in the rat was investigated. The studies were conducted with diets supplying fat up to levels which would approximate the percentage of fat calories supplied in human diets. These levels were selected to evaluate whether the typical response to meal-eating observed in the rat could be anticipated in human subjects consuming diets considerably higher in fat content. Male rats were fed diets containing 10, 20 or 30% fat, these levels supplying from 21 to 52% of the ingested calories. Increasing the level of fat from 10 to 30% appeared to facilitate the adjustment of food consumption in meal-fed rats. Following the first week of the experiment, during which meal-eaters fed the 10 or 20% fat diets lost weight, meal-fed animals gained weight at essentially the same rate as did nibbling (ad libitum-fed) rats, despite a reduced food intake. The activities of the enzymes studied (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme) and of fatty acid synthesis from acetate-1-^{14}C or glucose-U-^{14}C were increased by meal-feeding in rats fed diets containing 10 or 20% fat levels, but not in animals consuming 30% fat diets. The activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme and the lipogenic capacity decreased with increasing levels of dietary fat in adipose tissue of both meal-fed and nibbling rats. The significance of these observations in relation to the homeostatic control of lipid metabolism is discussed. It is suggested that the inability to demonstrate meal-feeding effects in human subjects may be due to the high level of fat in normal human diets.

The feeding of a single daily meal (meal-feeding) has been shown to induce marked adaptive changes in the rat. The most pronounced change observed is an increased ability to synthesize fatty acids. Tepperman and Tepperman (1) reported that liver slices from meal-fed rats incorporated significantly greater quantities of acetate-^{14}C and glucose-^{14}C into fatty acids than did similar preparations from control animals. This hyperlipogenic response to meal-feeding has also been observed in rat adipose tissue (2–4). The increase in lipid synthesis in both liver and adipose tissue of meal-fed rats is accompanied by increased activities of the pentose cycle enzymes, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (2, 5, 6), NADP-malic dehydrogenase (6, 7) and citrate cleavage enzyme (6). These enzymes are involved in the supply of substrate, acetyl-CoA, and reduced coenzymes for the support of lipogenesis (6, 8–10).

Most of the studies concerning meal-feeding have been carried out with diets high in carbohydrates. The hyperlipogenesis observed reflects the need of the organism to convert carbohydrate calories ingested during the meal period to a storage form, namely lipid, for the supply of energy during the period of fast between meals. In accord with this is the observation that no increase in lipogenesis or enzyme activity is observed in meal-fed rats consuming a "carbohydrate-free" diet (6, 11). Recent interest in periodicity of eating has centered around the possible significance of meal-eating in man (12). Studies with human subjects have not been nearly as definitive as animal studies (13–16). However, animal studies have not been conducted with diets comparable

Received for publication August 15, 1966.

[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

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in fat content to those consumed by human subjects; that is, with fat supplying approximately 40% of the caloric intake.

The present report describes the response to meal-feeding in adipose tissue of rats fed diets supplying from 20 to 50% of the ingested calories as fat. The data demonstrate that dietary fat levels in this range can markedly alter the lipogenic and enzymatic response to meal-feeding.

MATERIALS AND METHODS

Male rats of the Holtzman strain, weighing approximately 265 g, were used. They were housed in stainless steel cages having raised wire floors in a temperature and humidity controlled room (21° and 50% relative humidity). The animals were divided into 6 groups of 10 rats each in such a manner as to have similar initial body weights for each group. Three groups were allowed to consume the diets ad libitum (nibblers), and the other 3 groups were allowed access to the diet from 8:00 to 10:00 AM only (meal-eaters). The animals were maintained on their respective regimens for 4 weeks and body weight and food consumption were determined at weekly intervals. The composition of the diets used is shown in table 1; these diets were nutritionally adequate in all respects. The hydrogenated vegetable oil used was shown by gas chromatographic analysis to contain approximately 30% linoleate, which adequately met the requirement for essential fatty acids. The level of protein was varied to maintain an essentially constant calorie-to-protein ratio (20.8 kcal/g protein) and the cystine supplement was added at a level of 1.5% of the protein. The percentage of calories derived from fat was approximately 21, 38 and 52% for diets 1, 2 and 3, respectively.

TABLE 1
Composition of diets

	Diets		
	1	2	3
	%	%	%
Casein (vitamin-free)	20.0	22.5	24.9
L-Cystine	0.30	0.34	0.37
Vitamins [1]	2.2	2.2	2.2
Salt mixture [2]	4.0	4.0	4.0
Non-nutritive fiber	4.0	4.0	4.0
Fat [3]	10.0	20.0	30.0
Glucose	59.50	46.96	34.53

[1] The vitamin mixture supplied the following per 100 g of diet: vitamin A, 1980 units; vitamin D, 220 units; and (in milligrams) α-tocopherol, 11; ascorbic acid, 99; inositol, 11; choline·Cl, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; and (in μg) biotin, 4.4; folic acid, 198; vitamin B_{12}, 3 (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation, Cleveland).

[2] Salt mixture, USP XIV (Nutritional Biochemicals Corporation).

[3] Crisco, Procter and Gamble, Cincinnati.

At the termination of the 4-week experimental period, the meal-eating rats were killed immediately following the meal period, whereas the nibbling animals had access to food up until the time of killing. The rats were decapitated, exsanguinated and the epididymal adipose tissue was rapidly excised; one pad was used for metabolic studies and the other was homogenized for enzyme assay. Pieces of the thin peripheral portion of the fat pad, weighing approximately 100 mg, were used for incubation. The tissues were incubated in calcium-free Krebs-Ringer bicarbonate buffer (17),[3] pH 7.4, in a shaking water-bath (90 strokes/min) at 38° and under an atmosphere of 95% O_2 to 5% CO_2 for 3 hours. The incubations were carried out in 25-ml Erlenmeyer flasks fitted with a rubber serum stopper having a hanging polyethylene well containing a 2 cm × 2 cm piece of Whatman no. 1 filter paper. At the end of the incubation, the filter paper was saturated with 0.1 ml of 25% KOH added with a syringe and needle through the rubber stopper; in the same manner, 0.5 ml of 0.2 N H_2SO_4 was added to the incubation medium to stop the incubation and release the CO_2. To insure complete liberation and trapping of CO_2, shaking was continued for 20 minutes. The trapped radioactive CO_2 was assayed essentially as described by Buhler (18). The filter paper was transferred to a liquid scintillation vial, allowed to dry, was spread flat and 10 ml of toluene scintillation solution were added. Using standard $NaH^{14}CO_3$, this method was found to yield an overall efficiency of 20%.

The tissue was treated in a manner similar to that described by Cahill et al. (19). The tissue was rinsed in saline several times and transferred to a tube con-

[3] The insulin used in these studies was generously supplied by Dr. W. Bromer, Eli Lilly and Company, Indianapolis.

taining 15 ml of chloroform : methanol solution (2:1, v/v), and was extracted for 3 hours with continuous shaking. This procedure has been shown by gravimetric analysis to remove 98 to 99% of the lipids. The lipid extract was washed twice by the method of Folch et al. (20) to remove nonlipid radioactivity. After evaporation of the solvent under a stream of nitrogen, the lipids were saponified by refluxing at 80 to 85° in 3% methanolic KOH. Following saponification, the solution was diluted by the addition of one volume of water. The nonsaponifiable lipids were removed by 3 successive extractions with 5 ml of petroleum ether (BP 30–60°). The solution was acidified with HCl and the fatty acids were removed by 3 successive extractions with 5 ml of petroleum ether. The combined ether extract was backwashed with water and transferred to a liquid scintillation vial. The solvent was evaporated under a stream of nitrogen and dissolved in 10 ml of toluene scintillation solution. The volume of the aqueous phase containing glyceride-glycerol was determined and an aliquot was used for counting in 10 ml of toluene scintillation solution. The defatted tissue was transferred to a tube containing 2 ml of 25% KOH and was heated in a boiling water bath until digested. Alcohol was added to precipitate the glycogen which was refrigerated and allowed to stand overnight. The glycogen precipitate was centrifuged, the supernatant discarded and the precipitate was hydrolyzed with 1 N H_2SO_4. An aliquot of the hydrolysate was transferred to a scintillation vial and 10 ml of Bray's (21) scintillation solution were added. The toluene scintillation solution used contained per liter: 4 g 2, 5-diphenyloxazole (PPO), 0.015 g 1, 4-bis-2-(5-phenyloxazolyl) benzene (POPOP), 230 ml ethanol and toluene to one liter. All samples were counted in a Nuclear-Chicago Model 722 ambient temperature liquid scintillation spectrometer.

The epididymal fat pad used for enzyme assay was homogenized in 10 ml of 0.15 M KCl with a Virtis homogenizer. The homogenate was centrifuged at 1000 × *g* for 15 minutes and the clear intermediate layer was used for assay. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed spectrophotometrically as described by Glock and McLean (22). Malic enzyme (NADP-malic dehydrogenase) (EC 1.1.1.40) was assayed by the method of Ochoa (23). Nitrogen content of the homogenate was determined by micro-Kjeldahl digestion followed by Nesslerization (24).

The data were evaluated statistically by analysis of variance and intercell comparisons were made using Student's *t* test.

RESULTS

The body weight and food consumption data obtained in this study are shown in table 2. The meal-fed animals consuming the 10 and 20% fat diets lost weight during the first week, whereas those consuming the 30% fat-containing diet maintained their initial body weight during this adjustment period. The rate of gain after the first week was not markedly different for any of the groups studied, with the possible exception of the nibbling animals fed the 30% fat diet. In the meal-eating groups, the final body weights reflected largely the magnitude of the weight change during the first week on experiment. These changes in weight were closely correlated with alterations in food consumption (table 2). During the first week, meal-fed rats ate 47, 55 and 75% of the amounts consumed by nibbling rats fed the 10, 20 and 30% fat diets, respectively. After 4 weeks on these regimens, the meal-eating rats fed 10, 20 and 30% fat-containing diets consumed 79, 88 and 86%, respectively, of the amounts eaten by ad libitum-fed control animals. Therefore, these data imply that after 4 weeks on the meal-eating regimen, the animals had adapted in such a manner that weight gain was proceeding at a rate similar to that observed for nibbling control animals despite a reduced food intake for the meal-fed animals.

The ability of isolated adipose tissue to utilize acetate-1-^{14}C was studied, and the data are presented in table 3. The oxidation of acetate-1-^{14}C was not significantly influenced by meal-feeding at the 20 and 30% levels of dietary fat. However, tissue from meal-fed rats consuming

TABLE 2

Body weight and food consumption of meal-eating and nibbling rats fed diets containing varying levels of fat

Regimen	Dietary fat	Week of experiment				
		Initial	1	2	3	4
	%			Body weight, g		
Meal-eating	10	264[1]	228	242	257	275
	20	265	247	264	284	302
	30	265	268	287	306	322
Nibbling	10	265	283	309	331	343
	20	263	289	307	329	343
	30	261	293	321	340	358
				Food consumption, g/rat/week		
Meal-eating	10	—	61	102	103	112
	20	—	70	100	103	106
	30	—	88	94	90	89
Nibbling	10	—	131	137	145	141
	20	—	127	110	121	121
	30	—	117	106	102	103

[1] Values are means for 10 rats.

TABLE 3

Oxidation and incorporation into fatty acids of acetate-1-^{14}C by isolated adipose tissue of meal-eating and nibbling rats fed diets containing varying levels of fat [1]

Dietary fat	Regimen	Acetate-1-^{14}C incorporated into:	
		CO_2	Fatty acid
		mμmoles substrate incorporated/100 mg tissue/3 hr	
10	Meal-eater	373 ± 24[2]	841 ± 50
	Nibbler	245 ± 12	438 ± 28
20	Meal-eater	244 ± 15	553 ± 66
	Nibbler	232 ± 11	195 ± 21
30	Meal-eater	254 ± 8	204 ± 25
	Nibbler	287 ± 18	143 ± 28

[1] Incubations were carried out in Ca++-free Krebs-Ringer bicarbonate buffer containing per ml: 10 μmoles acetate, 5 μmoles glucose, 0.1 unit insulin and 0.167 μCi acetate-1-^{14}C.
[2] Mean for 5 rats ± SEM.

the 10% fat diet oxidized significantly more acetate than did tissue from nibbling animals ($P < 0.005$). Dietary fat did not alter the ability of tissue from nibbling rats to oxidize acetate, but the adipose tissue of meal-eating rats fed the 10% fat diet did produce more $^{14}CO_2$ from acetate-1-^{14}C than tissue from meal-fed rats consuming the 20 or 30% fat-containing diets ($P < 0.005$). Meal-feeding increased fatty acid synthesis in adipose tissue of rats fed the 10 or 20% fat diets ($P < 0.001$), but not in tissue of animals fed the 30% fat diet. In both meal-eating and nibbling rats, adipose tissue from animals fed the 10% fat diet incorporated more acetate-1-^{14}C into fatty acid than did tissue of animals fed diets containing 20 or 30% fat ($P < 0.001$). However, only in tissue from meal-fed animals was lipogenesis significantly decreased by increasing the level of dietary fat from 20 to 30% ($P < 0.005$). Although the amount of acetate-1-^{14}C incorporated into fatty acids was less for tissue of nibbling rats consuming 30%, as compared with 20% fat-containing diets, this difference was not statistically significant (table 3).

The ability of adipose tissue to utilize glucose-U-^{14}C in vitro was also studied as shown in table 4. The oxidation of glucose to CO_2 was significantly increased by meal-feeding in adipose tissue of rats fed the 10 or 20% fat diets, but not in

TABLE 4

In vitro utilization of glucose by adipose tissue from meal-eating and nibbling rats fed varying levels of fat [1]

Dietary fat	Regimen	Glucose-U-^{14}C incorporated into:			
		CO_2	Fatty acid	Glyceride-glycerol	Glycogen
%		*mμmoles substrate incorporated/100 mg tissue/3 hr*			
10	Meal-eater	1165 ± 127 [2]	1261 ± 144	318 ± 44	29 ± 4
	Nibbler	559 ± 81	445 ± 79	208 ± 10	25 ± 3
20	Meal-eater	712 ± 110	545 ± 85	185 ± 12	24 ± 2
	Nibbler	188 ± 7	75 ± 9	122 ± 9	15 ± 1
30	Meal-eater	224 ± 29	87 ± 25	129 ± 12	17 ± 4
	Nibbler	165 ± 24	45 ± 10	109 ± 11	12 ± 3

[1] Incubations were carried out in Ca^{++}-free Krebs-Ringer bicarbonate buffer containing per ml: 5 μmoles glucose, 0.1 unit insulin and 0.167 μCi glucose-U-^{14}C.
[2] Mean for 5 rats ± SEM.

TABLE 5

Activity of glucose 6-phosphate, 6-phosphogluconate and NADP-malic dehydrogenases in adipose tissue of meal-eating and nibbling rats fed varying levels of fat

Dietary fat	Regimen	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Malic enzyme
%		*μmoles substrate utilized/min/mg N*		
10	Meal-eater	0.933 ± 0.060 [1]	0.256 ± 0.023	1.210 ± 0.107
	Nibbler	0.334 ± 0.028	0.175 ± 0.008	0.316 ± 0.024
20	Meal-eater	0.420 ± 0.046	0.191 ± 0.016	0.550 ± 0.067
	Nibbler	0.207 ± 0.014	0.139 ± 0.008	0.120 ± 0.013
30	Meal-eater	0.187 ± 0.013	0.156 ± 0.009	0.136 ± 0.019
	Nibbler	0.188 ± 0.020	0.132 ± 0.006	0.093 ± 0.007

[1] Mean for 10 rats ± SEM.

animals consuming the 30% fat diet. Increasing the level of dietary fat decreased the ability of isolated adipose tissue to oxidize glucose in both meal-fed and nibbling animals except for nibbling rats fed the 20 as compared with the 30% fat diet. The incorporation of glucose into fatty acids, glyceride-glycerol and glycogen followed a similar pattern, being significantly increased by meal-feeding at the 10 and 20% fat levels, but not 30%, and decreased by increasing dietary lipid in both meal-eating and nibbling rats, except that the differences between tissue from nibblers fed 20 or 30% fat were not significant.

The activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme in adipose tissue of meal-eating and nibbling rats consuming the 10, 20 or 30% fat-containing diets are presented in table 5. These 3 enzymes were influenced in a similar way by the experimental variations studied. Meal-feeding significantly increased the activity of these enzymes in animals fed the 10 or 20% fat-containing diets ($P < 0.001$). However, at the 30% fat level, only the activity of 6-phosphogluconate dehydrogenase was higher in adipose tissue of meal-fed rats ($P<0.05$). The activity of the enzymes studied was reduced in tissue of both meal-eating and nibbling rats by increasing the level of dietary fat from 10 to 20%. Increasing the dietary fat level further, to 30%, resulted in a still greater decrease of glucose 6-phosphate dehydrogenase and malic enzyme in adipose tissue of meal-fed rats, but not of nibbling animals. The activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases were similar for both meal-fed and nibbling rats at the 30% fat level.

DISCUSSION

The results presented in this report demonstrate the marked effect of dietary fat on lipogenesis and on the response to meal-feeding. The effect of dietary fat on

lipogenesis in rat liver has received considerable attention. High fat-containing diets have been shown to markedly depress lipogenesis in liver, both in vitro (25, 26) and in vivo (27). The studies of Hill et al. (28) have demonstrated the extreme sensitivity of the hepatic lipogenic pathways to dietary lipid by showing that as little as 2.5% dietary fat would depress hepatic lipogenesis from acetate. The effect of dietary fat on lipogenesis in rat adipose tissue has been studied to a lesser extent. Hausberger and Milstein (29) observed that fatty acid synthesis from acetate by isolated adipose tissue was inhibited by feeding a high fat, low carbohydrate diet. These results have been confirmed by Leveille and Hanson (6) and Leveille (11). It has further been demonstrated that feeding a diet high in fat and almost free of carbohydrate abolished the lipogenic response to meal-feeding in adipose tissue (6) and the enzymic response in liver and adipose tissue (6, 11).

The possible implications of meal-feeding to obesity and heart disease in man have been of considerable concern since the human is basically a "meal-eater" (12). However, the data available concerning the effects of meal-eating as compared with nibbling in man are far from definitive. Cohn et al. (12) present a considerable amount of suggestive evidence implying that man responds to meal-feeding as does the rat. A similarity in response for man and the laboratory rat to a meal-eating regimen is also implied by the reports of Fabry et al. (13, 14). However, observations from this laboratory (15) and the recent report of Knittle (16) suggest that man is much less responsive to meal-eating than is the rat. The results of the present study demonstrate that levels of fat comparable to those used in human dietaries (38–50% of total calories derived from fat) markedly depress or completely inhibit the meal-feeding response in rat adipose tissue. The data demonstrate that both the enhanced rate of fatty acid synthesis from acetate-1-^{14}C and from glucose-U-^{14}C and the increased enzyme activities resulting from meal-feeding are inhibited by 30% dietary fat. These observations are in accord with results obtained in animals fed higher fat levels (6, 11) and may explain why man may be less responsive to meal-feeding than is the rat (15, 16).

The mechanism involved in the reduction of enzymatic and lipogenic activity by fat-feeding (or conversely by decreasing the dietary carbohydrate level) is not clear. Several reports have demonstrated that diet-induced increases in glucose 6-phosphate dehydrogenase activity are dependent upon the simultaneous ingestion of carbohydrate and protein (30–32). These observations have led to the speculation that carbohydrate or glucose is a specific "inducer" of this enzyme (32) and more recently Weber and Covery (33) have proposed that insulin is the specific agent responsible for the induction of glucose 6-phosphate dehydrogenase. However, the evidence supporting these proposals is not conclusive. A critical survey of the literature supports a different conclusion, that the changes in the activity of glucose 6-phosphate dehydrogenase (and malic enzyme) are secondary to alterations which increase the metabolic demand for reducing equivalents in the form of NADPH. Thus in fasting-refeeding (7) and in meal-feeding (34) the increase in glucose 6-phosphate dehydrogenase and malic enzyme activity is preceded by an increase in lipogenesis. These enzymes also increase in activity in the liver of rats fed diets containing hydrogenated fat rather than corn oil, with both diets containing identical amounts of glucose (35). Hepatic glucose 6-phosphate dehydrogenase activity is also increased by essential fatty acid deficiency in the mouse (36). The increase in enzyme activity induced by dietary saturated fat or by essential fatty acid deficiency cannot be explained by citing glucose (32) or insulin (33) as specific inducers of the enzyme, but in both of these situations there would be an increased demand for NADPH to support the enhanced fatty acid chain-length ening and desaturating activity (35). The increase in glucose 6-phosphate dehydrogenase observed in animals refed a high carbohydrate diet following a fast (5, 7, 8, 10, 30–32) or in insulin-treated animals (33) can also be explained on the basis of the increased demand for NADPH

to support the increase in lipogenesis induced by these treatments (7, 10, 37).

This proposed mechanism is also consistent with the decreased enzyme activity observed in the present study in animals fed increasing levels of fat. Dietary fat can rapidly inhibit lipogenesis (38) with a resulting decrease in the oxidation of NADPH which could in turn trigger a reduction in glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme activity (8–11, 31). Such a mechanism is in keeping with present concepts concerning the homeostatic control of lipogenesis and related requirements for reduced coenzymes (34).

The results obtained for food consumption and body weight are noteworthy. The observation that food consumption of meal-fed rats is more rapidly adjusted as the level of dietary fat increases is in keeping with the report of Smith et al. (39) and a previous report from this laboratory (11). The reasons for the more rapid adjustment are not clear. The results presented also suggest, as reported earlier (4), that meal-fed animals utilize their diet more efficiently. Following an initial adjustment to the meal-eating regimen, meal-fed rats gained weight at approximately the same rate as ad libitum-fed controls, despite a reduced food intake. This phenomenon appeared to be independent of dietary fat since growth rate was similar for meal-eating and nibbling animals at each dietary fat level after the first week of the experiment.

ACKNOWLEDGMENTS

The author expresses his appreciation to J. Taubr, L. Schiff and J. Heidker for technical assistance, to G. Isaac for statistical evaluation of the data, to B. James for care of the animals used and to Mrs. M. Iverson and Mrs. G. Castor for assistance in the preparation of the manuscript.

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Influence of Dietary Carbohydrates on Liver Content and on Serum Lipids in Relation to Age and Strain of Rat

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ABSTRACT To determine the effect of kind of carbohydrate on the lipid metabolism of the rat as influenced by age and heredity, rats of the BHE and Wistar strains, known to differ in their lipid metabolism, were fed high cholesterol diets identical except for the dietary carbohydrate, supplied as sucrose, cornstarch, or glucose. Livers of 150- and 350-day-old rats were analyzed for moisture, protein, total lipid, cholesterol, and glycogen; blood for total serum cholesterol and for noncholesterol lipids as fatty acids. The most striking differences were in liver lipids. Cholesterol and noncholesterol lipids were generally higher in livers of sucrose-fed rats of either strain than in dextrose- or cornstarch-fed rats. Livers of BHE rats contained more cholesterol and noncholesterol lipids than those from Wistar rats. Liver lipids of sucrose fed BHE rats increased with age; no significant age effect was apparent with cornstarch or glucose. With Wistar rats, liver cholesterol decreased with age with all 3 carbohydrates; noncholesterol lipids remained relatively constant. Differences in liver size and composition between fasted and nonfasted rats suggest that the metabolic activity of this organ varies with dietary carbohydrate. Serum cholesterol levels increased with age, were higher in BHE than in Wistar rats and did not differ significantly with dietary carbohydrate. Noncholesterol lipids were generally high in sera from sucrose-fed rats.

Investigations in this laboratory (1) have demonstrated differences in rate and average age of death of rats even when the diets fed supplied nutrients in amounts currently considered adequate. When a diet containing 25% dried egg was fed to rats of the BHE strain, average age at death was reduced and degenerative changes in the tissues were accelerated in comparison with the results when a diet containing no egg was fed. No such differences in response were observed, however, between the same 2 diets when fed to rats of the Wistar strain. Moreover, when a diet consisting of 100% egg was fed to BHE rats, survival was similar to that observed for this strain of rat fed the diet containing no egg, indicating that the shortened lifespan observed with the 25% egg diet resulted not from egg alone but from an interaction with other dietary ingredients. Sucrose, present in the 25% egg diet at a concentration of 39% was lacking in the 100% egg diet.

Investigations were undertaken, therefore, to determine the extent to which dietary carbohydrate and heredity were factors in the response to this high cholesterol diet. The response to the diets under investigation was determined for both BHE and Wistar rats, 2 strains differing significantly in their lipid metabolism (2). The present report deals with the composition of the livers and the serum lipids of BHE and Wistar rats fed the diet containing 25% egg with the carbohydrate supplied as sucrose, cornstarch or glucose. Other phases of this research will be presented in separate reports dealing with the influence of dietary carbohydrate on food intake, body weight and composition, serum proteins, mineral metabolism and survival and pathology of these rats.

EXPERIMENTAL

Three experimental diets were fed, identical in all respects except for the kind of carbohydrate. These diets contained the

Received for publication September 1, 1966.

following ingredients: (in grams/100 g) dried cooked egg, 25; casein, 12; lactalbumin, 6; hydrogenated vegetable oil,[1] 6; brewer's yeast, 7.5; modified Osborne and Mendel salt mixture (3), 3; cellulose flour, 1.5; and carbohydrate provided as sucrose, cornstarch or glucose, 39. Each rat received weekly 2 drops percomorph oil equal to approximately 400 IU vitamin A and 56 IU vitamin D daily and 36 mg α-tocopheryl acetate in 0.1 ml cottonseed oil (5.1 mg daily). In addition, 10 g fresh kale were fed twice weekly. The method of preparation and the nutrient composition of these diets are described in detail elsewhere (1).

The 2 strains of rats (Wistar and BHE) used in these experiments have been maintained in this laboratory for several generations under the same environmental conditions. The stock animals and the experimental animals were housed in air conditioned animal quarters kept as closely as possible at 27° and 45% relative humidity, with lighting regulated to give 12 hours of uniform illumination and 12 hours of darkness each day. The experimental animals, all male, were caged individually and fed ad libitum from weaning until killing, with water available at all times. For each diet, one group of rats was scheduled for killing at 150 days of age, a second at 350 days of age and a third was maintained with the diet until the animals died or death appeared to be imminent. Approximately one-half of the animals killed at 150 and at 350 days of age were killed without a preliminary fasting period; with the other half, food was removed from the cages approximately 17 hours before killing.

Animals were killed between 9 and 11 AM to minimize possible differences due to food consumption. They were anesthetized with Amytal (sodium) solution and blood was removed by heart puncture. Livers were removed, blotted free of excess blood and weighed immediately. A portion of the median lobe (approximately 20% of the total liver weight) was saved for histological examination, approximately one gram from the left lobe was removed for glycogen analysis and the remaining liver weighed and kept frozen until analysis was performed.

The portion of the liver saved for chemical analysis was homogenized, and weighed aliquots were analyzed for moisture by drying in a vacuum oven at 70°, for nitrogen determination by the macro-Kjeldahl method (4), for total lipids by a modification of the AOAC acid hydrolysis method (5) and for cholesterol by the method of Koval (6) following extraction with 0.1 M potassium acetate in absolute ethanol. The samples for glycogen determination were analyzed immediately upon removal of the liver (7). Blood was analyzed for total cholesterol (6). A limited number of serum samples was analyzed for noncholesterol lipids as total fatty acids using a method that combined procedures described by Mendelson (8) and Albrink (9). The method consisted of simultaneous precipitation of serum protein, extraction of lipid, saponification, acidification, extraction of fatty acids and colorimetric determination of the extractable acids. Statistical analyses of the results were made by the *t* test as described by Snedecor (10).

RESULTS

Table 1 presents data for 150- and 350-day-old BHE and Wistar rats that were killed after an overnight fast. Data for body weight are included as evidence that the differences reported cannot be attributed to body weight. The results for the liver are presented as total content; the values for noncholesterol lipids were obtained by subtracting cholesterol from total lipid content.

At 150 days of age, the livers from fasted BHE rats fed sucrose were significantly heavier ($P < 0.05$) than those from rats fed glucose. The composition of the livers of these rats did not differ significantly with the kind of carbohydrate consumed and differences in total content were a reflection of liver size. Liver glycogen and serum cholesterol were uninfluenced by dietary carbohydrate. The data for noncholesterol lipids in the sera of these rats were too limited to establish significant differences.

[1] Crisco, Procter and Gamble, Cincinnati; the Crisco available at the time this study was initiated contained approximately 7% linoleic acid and was used throughout the investigation.

TABLE 1

Influence of dietary carbohydrate on liver content and serum lipids of 150- and 350-day-old fasted rats of the BHE and Wistar strains

Dietary carbohydrate	No. of rats	Body wt [1]	Liver wt	Total liver content					Serum	
				H_2O	Protein	Cholesterol	Non-cholesterol lipid	Glycogen	Cholesterol	Non-cholesterol lipid [2]
		g	*g*	*g*	*g*	*mg*	*g*	*mg*	*mg/100 ml*	*mg/100 ml*
BHE rats, 150 days										
Sucrose	10	485 ± 14 [3]	17.3 ± 1.0	10.5 ± 0.5	3.63 ± 0.15	586 ± 125	2.10 ± 0.29	98 ± 14	87 ± 9	232 ± 8 (3) [4]
Cornstarch	12	463 ± 17	15.5 ± 0.8	9.4 ± 0.5	3.32 ± 0.16	453 ± 60	1.80 ± 0.19	102 ± 9	79 ± 4	185 ± 8 (3)
Glucose	11	469 ± 12	14.8 ± 0.7	9.3 ± 0.4	3.16 ± 0.13	404 ± 83	1.42 ± 0.17	83 ± 11	84 ± 5	208 ± 4 (3)
Wistar rats, 150 days										
Sucrose	10	511 ± 11	16.1 ± 0.6	10.4 ± 0.4	3.66 ± 0.11	251 ± 23	1.30 ± 0.09	118 ± 26	93 ± 8	226 ± 23(6)
Cornstarch	11	473 ± 13	13.5 ± 0.6	8.9 ± 0.4	3.08 ± 0.12	172 ± 27	0.89 ± 0.08	89 ± 22	82 ± 4	155 ± 16(6)
Glucose	11	485 ± 15	13.6 ± 0.5	9.0 ± 0.4	3.13 ± 0.12	130 ± 16	0.84 ± 0.06	80 ± 5	83 ± 5(10)	191 ± 16(5)
BHE rats, 350 days										
Sucrose	12	628 ± 17	25.3 ± 1.2	15.1 ± 0.8	4.67 ± 0.18	1369 ± 102	3.28 ± 0.32	144 ± 41(5)	276 ± 33(11)	662 ± 78(5)
Cornstarch	6	558 ± 21	19.2 ± 1.8	12.1 ± 1.1	4.00 ± 0.29	648 ± 188	1.95 ± 0.38	124 ± 43	203 ± 43	606 ± 135
Glucose	5	531 ± 23	18.3 ± 0.4	11.9 ± 0.2	3.84 ± 0.08	384 ± 88	1.66 ± 0.20	99 ± 16	203 ± 42	627 ± 117
Wistar rats, 350 days										
Sucrose	6	598 ± 16	16.0 ± 0.4	10.6 ± 0.2	3.60 ± 0.09	172 ± 29	1.23 ± 0.11	83 ± 9	105 ± 3	286 ± 27
Cornstarch	8	550 ± 13	13.8 ± 0.5	9.3 ± 0.3	3.26 ± 0.11	83 ± 14	0.86 ± 0.06	72 ± 16	122 ± 14	286 ± 38
Glucose	9	582 ± 25	14.3 ± 0.7	9.8 ± 0.5	3.10 ± 0.14	78 ± 11	0.90 ± 0.05	89 ± 14	101 ± 5	291 ± 12

[1] Weight before 17-hour fast.

[2] Expressed as stearic acid.

[3] SE of mean.

[4] Numbers in parentheses indicate number of rats when different from that in column 2.

The livers from 150-day-old Wistar rats fed sucrose were significantly heavier ($P < 0.01$) than those from rats fed cornstarch as well as from those receiving glucose. The livers of sucrose-fed rats also contained more protein, cholesterol and noncholesterol lipids than those of cornstarch- or glucose-fed rats, with the differences highly significant ($P < 0.01$) except for cholesterol in the livers of cornstarch-fed animals ($P < 0.05$). In this strain of rats, the percentage of protein was lower ($P < 0.05$) and the percentage of cholesterol and noncholesterol lipids higher ($P < 0.05$) in the livers of sucrose-fed rats than in either constarch- or glucose-fed animals and the differences in content observed were not related entirely to liver size. As with the BHE rat, neither liver glycogen nor serum cholesterol was influenced by dietary carbohydrate. Serum fatty acids were significantly higher ($P < 0.05$) when the diet contained sucrose than when it contained cornstarch.

At 350 days of age, differences in the response of BHE and Wistar rats to dietary carbohydrate were in general similar to those observed with 150-day-old rats. Livers of sucrose-fed rats were consistently heavier and contained more protein, cholesterol and noncholesterol lipids than those of cornstarch- or glucose-fed rats. The differences between sucrose and glucose were highly significant ($P < 0.01$) for both strains of rats. In the BHE rats, however, the results with cornstarch tended to be intermediate between those with sucrose and glucose, whereas in the Wistar rats the results were similar to those for glucose. In both strains of rats differences in liver content resulted not only from differences in liver weight but also from differences in percentage composition. Neither liver glycogen nor serum lipids differed significantly with the kind of carbohydrate fed.

The response of BHE and Wistar rats to dietary carbohydrates was qualitatively similar in many respects. Highly significant differences between these 2 strains were observed, however, in liver size and in cholesterol and noncholesterol lipid concentration and content, particularly when comparisons were based on the response to identical diets. Of interest were the differences in the response of these 2 strains to age. By 350 days, the livers of BHE rats become heavier than those from 150-day-old rats fed comparable diets, whereas no significant change with age occurred in the size of the livers of Wistar rats. Liver cholesterol and noncholesterol lipids increased ($P < 0.01$) with age in sucrose-fed BHE rats but did not change significantly in cornstarch- or glucose-fed rats. In contrast, liver cholesterol of Wistar rats decreased significantly ($P < 0.01$) with all 3 carbohydrates; noncholesterol lipids were not influenced by age. Cholesterol and noncholesterol lipids increased significantly ($P < 0.01$) in the sera of BHE rats. Cholesterol and noncholesterol lipids in the sera of Wistar rats were also higher in the older animals but remained significantly lower ($P<0.01$) than in comparable BHE rats.

Table 2 summarizes the results for nonfasted rats, as compared with those presented in table 1 for fasted animals. In contrast with the results for 150-day-old fasted rats, there were no significant differences related to dietary carbohydrate in liver weight or in protein, cholesterol or noncholesterol lipid content of the livers of either BHE or Wistar rats, although there was a consistent tendency for high values in the BHE rats fed the sucrose-containing diet. The glycogen content of the livers of these young BHE rats tended to be high with sucrose but the differences failed to meet the test for significance ($P < 0.05$). The glycogen content of the livers of Wistar rats was significantly higher when the diet contained sucrose than when the carbohydrate was cornstarch ($P < 0.05$) or glucose ($P < 0.01$). No consistent differences related to carbohydrate were observed in the serum cholesterol level of either strain of rats but noncholesterol serum lipids were consistently higher in sucrose-fed rats than in either cornstarch- or glucose-fed animals.

By 350 days carbohydrate differences were apparent and were similar in many respects to the differences observed in the fasted rats. Liver weights, however, and cholesterol content of the livers of the cornstarch-fed BHE rats were similar to those of the sucrose-fed rats and differed

TABLE 2

Influence of dietary carbohydrate on liver content and on serum lipids of 150- and 350-day-old nonfasted rats of the BHE and Wistar strains

Dietary carbohydrate	No. of rats	Body wt	Liver wt	Total liver content					Serum	
				H_2O	Protein	Cholesterol	Non-cholesterol lipid	Glycogen	Cholesterol	Non-cholesterol lipid [1]
		g	*g*	*p*	*g*	*mg*	*g*	*mg*	*mg/100 ml*	*mg/100 ml*
				BHE rats, 150 days						
Sucrose	10	460 ± 16 [2]	20.1 ± 0.9	12.3 ± 0.5	3.85 ± 0.18	582 ± 92	2.24 ± 0.23	580 ± 64	113 ± 9	511 ± 166(5) [3]
Cornstarch	11	449 ± 15	18.6 ± 0.8	11.7 ± 0.6	3.68 ± 0.13	496 ± 53	1.65 ± 0.13	475 ± 41	110 ± 11	290 ± 38 (5)
Glucose	11	469 ± 15	18.3 ± 0.8	11.6 ± 0.4	3.62 ± 0.13	414 ± 94	1.52 ± 0.18	459 ± 27	101 ± 8(10)	273 ± 43 (5)
				Wistar rats, 150 days						
Sucrose	11	502 ± 16	19.2 ± 0.8	12.7 ± 0.6	3.84 ± 0.14	197 ± 15	1.19 ± 0.07	813 ± 40	113 ± 7	410 ± 60 (5)
Cornstarch	11	508 ± 13	18.9 ± 0.8	12.6 ± 0.5	3.86 ± 0.15	235 ± 48	1.21 ± 0.13	671 ± 56	105 ± 7(10)	279 ± 14 (5)
Glucose	11	502 ± 15	18.1 ± 0.7	12.1 ± 0.4	3.65 ± 0.14	179 ± 25	1.12 ± 0.12	633 ± 39	108 ± 6	297 ± 18 (6)
				BHE rats, 350 days						
Sucrose	7	582 ± 24	24.5 ± 1.9	15.0 ± 0.9	4.26 ± 0.26	930 ± 212	3.06 ± 0.50	733 ± 204(3)	186 ± 21	633 ± 51 (3)
Cornstarch	6	567 ± 31	24.3 ± 2.3	15.3 ± 1.3	4.51 ± 0.38	1047 ± 244	2.38 ± 0.33	656 ± 91	231 ± 44	809 ± 158
Glucose	5	565 ± 12	20.0 ± 1.1	13.4 ± 0.8	3.96 ± 0.17	254 ± 54	1.44 ± 0.08	571 ± 81	164 ± 26	497 ± 73
				Wistar rats, 350 days						
Sucrose	8	596 ± 26	20.6 ± 1.0	13.8 ± 0.7	4.10 ± 0.19	158 ± 21	1.32 ± 0.12	810 ± 48	151 ± 11	660 ± 48
Cornstarch	7	572 ± 10	17.1 ± 0.5	11.6 ± 0.4	3.54 ± 0.12	88 ± 6	0.90 ± 0.05	594 ± 51	129 ± 4	355 ± 41
Glucose	9	549 ± 9	17.0 ± 0.6	11.6 ± 0.4	3.50 ± 0.11	84 ± 11	0.88 ± 0.05	581 ± 37	126 ± 9	362 ± 30

[1] Expressed as stearic acid.

[2] SE of mean.

[3] Numbers in parentheses indicate number of rats when different from that in column 2.

significantly ($P < 0.01$) from those of the glucose-fed rats. Noncholesterol lipids and glycogen content of the livers of cornstarch-fed animals tended to be intermediate between the values for sucrose- or glucose-fed rats but did not differ significantly from either. In the Wistar rat fed sucrose, liver weight, protein, cholesterol, noncholesterol lipids and glycogen content of the liver were all significantly higher ($P < 0.01$) than in cornstarch- or glucose-fed rats. In serum also, cholesterol tended to be high when the diet contained sucrose, and noncholesterol lipids were significantly higher ($P<0.01$) than with cornstarch or glucose.

Liver weight ($P < 0.01$) and liver cholesterol ($P < 0.05$) increased significantly with age in nonfasted BHE rats fed sucrose or cornstarch but did not change significantly in glucose-fed rats. Cholesterol and noncholesterol levels in the sera of these rats were also consistently higher in the older rats, with the differences significant ($P < 0.01$) except for the noncholesterol lipids in the serum of sucrose-fed rats. In 350-day-old nonfasted Wistar rats liver weights did not differ significantly from those observed in 150-day-old rats fed comparable diets. Liver cholesterol decreased with age ($P < 0.01$) when the carbohydrate was cornstarch or glucose but did not change significantly when the diet contained sucrose. Cholesterol and noncholesterol lipids in the sera of these rats showed a consistent increase with age with all 3 carbohydrates.

Differences in the response of fasted and nonfasted rats to dietary carbohydrate appeared to be the result of differences in the metabolic activity of the liver when dietary carbohydrate was varied. Thus, in the 150-day-old Wistar rat fed sucrose, the significant loss in liver weight that occurred as the result of an overnight fast was due chiefly to loss of water and glycogen, with the liver cholesterol content higher in the fasted than in the nonfasted rat. When the carbohydrate was cornstarch or glucose, loss in liver weight was accompanied by decreased protein, cholesterol and noncholesterol lipids as well as by loss of water and protein. In the 350-day-old BHE rat fed sucrose, only glycogen was lost as the result of the overnight fast. Liver weight, liver water, protein and noncholesterol lipids remained unchanged and, again, liver cholesterol content was higher in the fasted than in the nonfasted rat. In contrast, whereas the livers of the 350-day-old nonfasted BHE rats fed cornstarch were large, those of the fasted animals were reduced significantly and showed loss of water and glycogen as well as consistently smaller amounts of all other components, protein, cholesterol and noncholesterol lipids.

Despite the large loss of weight before the death of rats kept for survival,

TABLE 3

Influence of dietary carbohydrate on survival and on liver and serum lipid of rats of the BHE and Wistar strains

Dietary carbohydrate	No. of rats	Age at death	Wt loss before death	Total liver content			Serum cholesterol
				Wt	Cholesterol	Non-cholesterol lipid	
		days	*g*	*g*	*mg*	*g*	*mg/100 ml*
				BHE rats			
Sucrose	10	444(38) [1]	203	20.7	815 ± 243 [2]	1.53 ± 0.26	281 ± 29(5)
Cornstarch	13	595	157	22.9	441 ± 136	1.65 ± 0.41	292 ± 12(6)
Glucose	15	543	153	19.8	241 ± 76	1.28 ± 0.25	232 ± 61(9)
				Wistar rats			
Sucrose	6	583(32)	63	18.4	108 ± 31	0.91 ± 0.14	138 ± 19(5)
Cornstarch	7	636	94	16.9	69 ± 9	0.96 ± 0.14	94 ± 22(5)
Glucose	8	565	123	16.5	92 ± 41	1.00 ± 0.23	102 ± 17(6)

[1] Numbers in parentheses indicate number of rats when different from that in column 2. Data on age of death includes additional longevity animals fed the sucrose diet.
[2] SE of mean.

significant differences due to carbohydrate and to strain were still apparent (table 3). Average age of death of BHE rats fed sucrose was significantly lower than that of Wistar rats fed the same diet and than that of either strain fed cornstarch or glucose. Liver cholesterol content still remained higher in BHE rats when the diet contained sucrose than when the diet contained cornstarch or glucose. Noncholesterol lipids then showed no differences related to carbohydrate. Serum cholesterol levels were high with all 3 carbohydrates. In the longevity Wistar rats, however, no carbohydrate differences were apparent, with relatively low levels of liver cholesterol, of liver noncholesterol lipids and of serum cholesterol.

DISCUSSION

The results reported here add to the increasing evidence that response to diet may be significantly influenced by the kind of dietary carbohydrate. A report by Hodges and Krehl (11) covers some of the most recent papers dealing with this subject.

Several possible explanations for differences in response to type of dietary carbohydrate have been suggested. Delay in absorption of glucose from starch caused by the need for preliminary hydrolysis of the polysaccharide does not appear to provide an explanation of the results reported here, considering the similarity noted frequently in the response of BHE and Wistar rats to cornstarch and glucose.

The results with BHE and Wistar rats indicate differences in the ability of these rats to adapt to a high cholesterol diet with age, but differences in liver lipids with the kind of dietary carbohydrate were still apparent at 350 days. The suggestion that differences in response to carbohydrate are transient (12), therefore, does not apply under the conditions of this report.

The possibility exists that bacterial flora may contribute to the lipid metabolism of animals fed different kinds of carbohydrate through their influence on bile acid excretion (13–15). The marked differences in the response of the 2 strains of rats to fasting, to carbohydrate, and to aging, however, suggest that the metabolic activity of the liver of these rats may be a more important factor in the response observed.

Ashworth et al. (16) have suggested that differences in the mechanism of blood lipid clearance by liver cells may have a bearing on the occurrence of persistent lipemia and upon the development of atherosclerosis in humans. The contrast in the results reported here for the change with age in serum lipids of BHE and Wistar rats suggests that differences in the response of these 2 strains may be due in part at least to a decrease with age in the ability of the liver of BHE rats to remove lipids from the blood. The ability of the liver to convert cholesterol to bile acids (17) may also be a contributing factor.

Perhaps more pertinent to the problem may be the influence of the kind of carbohydrate on the relative participation of different pathways in carbohydrate metabolism (18, 19) which may in turn influence the accumulation of liver lipids and the ability of the liver to handle the increased load. According to Siperstein and Fajan (20), lipogenesis and cholesterolgenesis may be accelerated by conditions that stimulate the hexose-monophosphate shunt. Preliminary results [2] from this laboratory have provided evidence for higher levels of glucose 6-phosphate dehydrogenase in the livers of sucrose-fed rats than in those of starch-fed animals, with higher levels of this enzyme in the liver of BHE rats than in those of Wistar rats fed comparable diets.

A factor that undoubtedly contributes to some of the differences in the response of BHE and Wistar rats is the tendency of the former to nephrosis (21), a condition commonly accompanied by hyperlipemia (22). From the results to date, it is not possible to establish whether the high liver lipids and the metabolic activity of the livers were primary factors in the acceleration of kidney disease and early death in BHE rats fed sucrose or whether the adverse effect of this diet was due

[2] Wu Chang, M. L., and M. Adams 1965 Influence of heredity and of dietary carbohydrate on some of the carbohydrate-metabolizing enzymes in the tissues of the young rat. Federation Proc., 24: 220 (abstract).

chiefly to its influence on the kidney and secondarily on the metabolic activity of the liver. It is apparent, however, that survival of BHE rats can be good with high serum cholesterol levels as well as with higher than normal liver lipids when the dietary carbohydrate is starch or glucose. There appears to be no simple answer to the cause for differences in metabolic response observed and it is likely that we are dealing with an interaction of several factors.

ACKNOWLEDGMENT

We wish to thank Mrs. Mamie E. Flowers for her assistance in calculating and summarizing the results included in this report.

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