Metabolic Derangements in Response of Rats to Ingestion of Imbalanced Amino Acid Mixtures

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ABSTRACT Histidine-U-^{14}C and threonine-U-^{14}C were used in tracer quantities to compare the effect of histidine imbalance on their metabolic behavior. Three groups of rats (six rats per group) were fed basal, histidine-imbalanced, and corrected diets. Two rats from each group were killed 2, 4 and 6 hours after feeding each diet. Radioactivity was determined in expired CO_2, urine and feces as well as in protein and nonprotein fractions of rat tissues. The experiment was done twice, once with histidine-U-^{14}C, and once with threonine-U-^{14}C to permit comparison between the behavior of limiting and nonlimiting amino acids in the imbalanced diet groups and their respective control groups (basal and corrected). Radioactivity losses from histidine in expired CO_2, urine and feces, and its incorporation into lipid fractions were lower in rats fed the imbalanced diet. The corresponding losses and incorporation values for threonine were higher. After 6 hours, radioactivity values in the TCA-soluble fractions of the tissues of rats fed the imbalanced diet containing labeled histidine were significantly lower than those of the control groups; in the protein fractions the reverse was true. Nonlimiting threonine behaved in an opposite manner. Radioactivity in both histidine and threonine isolated from hydrolyzed liver protein of the imbalanced diet groups was increased. However, significant differences in retention efficiency and efficiency of utilization of these amino acids were demonstrated; both parameters were higher for histidine and lower for threonine in the imbalanced diet groups. These results indicate enhanced protein synthesis, and validate Harper's hypothesis (1, 2) that the limiting amino acid is utilized with higher efficiency in protein synthesis subsequent to ingesting imbalanced amino acid mixtures.

The term amino acid imbalance was advanced and used to describe dietary amino acid patterns which result in depressed food intake and retarded growth. Alleviation of these effects could be achieved by adding small amounts of the most limiting indispensable amino acids in the diet (1, 2). To put the imbalance phenomenon in epidemiological terms: the causative agent is known and the symptoms are already established, but the sequence of events that leads to the development of these symptoms is unknown. Many investigators have directed their efforts to understanding and clarifying these events, and many hypotheses have been put forward and tested. The most promising hypothesis was proposed by Harper (1) and Harper et al. (2). In addition, they suggested short-term experiments to study the biochemical changes observed in tissues of protein-depleted rats after feeding an imbalanced diet, and to clear the controversy over the efficiency of utilization of the most limiting amino acid.

The following experiments were designed mainly to study the metabolic bases for the gross effects of amino acid imbalance in very short-term studies. In addition, the efficiency of utilization of the limiting and nonlimiting indispensable amino acids was evaluated.

The metabolic behavior of histidine-U-^{14}C was studied and compared when used in tracer quantities and fed to rats in basal, histidine-imbalanced and corrected diets with 10, 15 and 15% protein levels, respectively. Also, data were obtained to clarify the effect of imbalance on the metabolic fate of the indispensable and nonlimiting amino acids. Threonine was studied as an example of the response of a nonlimiting essential amino acid in experiments where threonine-U-^{14}C was added to the same basal, histidine-imbalanced and corrected diets and fed to rats. These

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experiments are closely related and complementary to the studies reported by Hartman and King (3). They tested this particular imbalance system using ^{15}N labeled histidine.

MATERIALS AND METHODS

In these experiments the excretory patterns of the animals in urine, feces and expired air were measured, and quantitative recovery of ingested isotope was achieved. For this purpose, special metabolism cages were designed and assembled. One-quart wide-mouth Mason jars were used as chambers. The animal was placed in this chamber on a floor made of 0.64-cm stainless steel screen supported by transverse glass rods glued to the jar walls with epoxy resin. Below the floor was a fine window screen to separate the feces from the urine. The latter was collected in the bottom of the chamber. Fixed near the mouth of the jar and at the lower part of the chamber is a piece of metal sheet to function as a food waste collector preventing contamination of urine with spilled food. The rear of the cage was covered with black paint to provide the rats with dark shelter during the day. Four holes were bored in the cap. Three of these were located in a row across the top. Each was 7 mm in diameter to accommodate aluminum tubes for water and for the inlet and outlet air. The fourth hole was located in the center being 2.5 cm in diameter, to function as an inlet to the feeder cup where the diet under investigation could be placed (fig. 1). The chamber was supplied with air from which moisture and CO_2 had been removed by passage through soda lime column packings. The air was drawn through the metabolism chamber and then bubbled through the alkaline-absorption tower where expired CO_2 was trapped for subsequent radiotracer analysis. The expired air was expelled through the suction pump. A bank of 12 cages was operated as a system. Each cage was provided with its separate alkaline-absorption tower, and drew its input air from a main supply line which had three inlets guarded with three soda lime column packings. The air was circulated into the system by the negative pressure created by the suction pump. Muffling of pump noise was achieved by attaching cotton baffles to the inlet and outlet of the suction pump. Behavior of the animals in these cages was tested by feeding 20% casein diets for 7 days, and it was found satisfactory.

The composition of the chemically defined diets used in these experiments was developed and tested in our laboratory based on the work of Sauberlich (4, 5). Tables 1 and 2 show the composition of the purified amino acid mixtures used in the various diets and the composition of the basal, histidine-imbalanced and corrected diets. Uniformly labeled histidine or threonine was added to the different diets to provide about 3 μCi/g diet ingested.

Male weanling rats of the Sprague-Dawley strain, weighing about 50 g, were used. Rats were placed individually in the metabolism cages supplied with water ad libitum and were fed the basal diet twice a day for a 1-hour interval for 10 days. This period served to train the animals to consume all their daily food in these two intervals and to condition them to the new environment and the purified diets.

Thirty-six rats were grouped according to weight into 18 groups. Each of the basal, imbalanced and corrected diets, labeled either with histidine or threonine was offered to three groups after a 12-hour starvation period. The diet was removed after 1 hour. Two rats were killed after 2-, 4- and 6-hour intervals.

Before the rats were killed, they were weighed and anesthetized with chloroform. Blood was collected by heart puncture. Following this, intestinal tract, liver, kidneys and left gastrocnemius muscle were removed rapidly; they were weighed and homogenized in 10% (w/w) trichloroacetic acid solution (TCA). The intestinal tract was washed of its contents with physiological saline before weighing and homogenization. The well-formed feces in the lower part of the intestinal tract and cecum contents were combined with the collected feces and homogenized, measured and centrifuged. The supernatant was stored for ^{14}C determination. After killing, the urinary bladder was emptied,

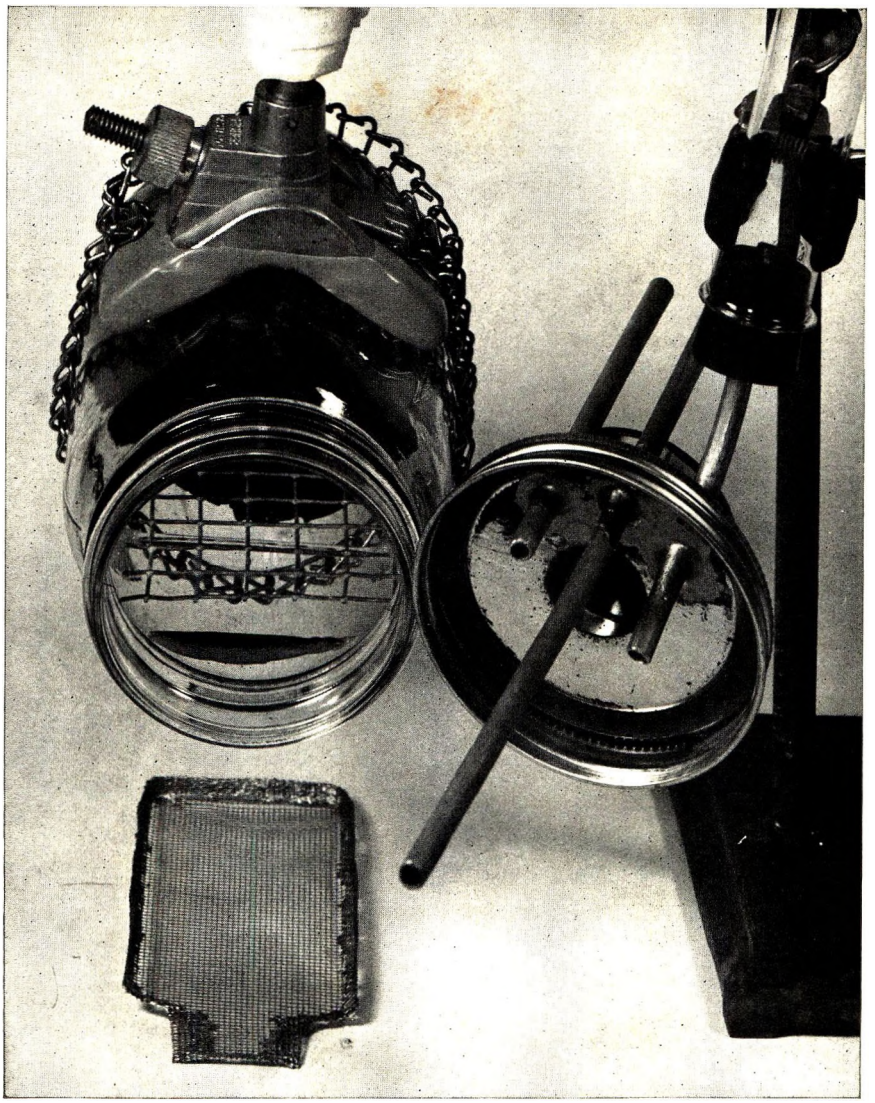

Fig. 1 Detailed picture of metabolism cage.

and its contents were added to the excreted urine; all the urine was diluted and stored.

The tissues were processed and prepared for counting by centrifuging the homogenates. The supernatants were decanted, and the protein-containing pellets were washed twice with hot TCA solution (85 to 90°). The washings were combined and saved.

Protein-containing pellets were suspended three times in hot 95% ethanol followed by three extractions with warm (40 to 45°) 1:1 ether–acetone mixture, then dried.

One-hundred-milligram portions of the dried protein fractions were autoclaved with 6 N HCl for 20 hours (121°, 15 lb/in^2). The hydrolysates were filtered and stored for counting except those of liver samples. Liver protein hydrolysates were divided into two portions; one portion was stored for later estimation of the total count, while the other portion was brought

TABLE 1

Composition of purified amino acid mixture used in various diets

	Basic amino acid mixture [1]	Imbalancing amino acid mixture [2]
	g/kg	*g/kg*
L-Alanine	3.00	
L-Arginine·HCl	4.00	4.00
L-Aspartic acid	3.00	
L-Asparagine·H_2O	3.00	
L-Cystine	1.50	1.50
L-Glutamic acid	20.00	
L-Glycine	2.00	
L-Histidine·HCl	2.50	
L-Leucine	10.00	10.00
L-Isoleucine	7.50	7.50
L-Lysine·HCl	9.25	
L-Methionine	4.00	4.00
L-Phenylalanine	3.25	3.25
L-Proline	2.50	
L-Serine	2.50	
L-Threonine	3.75	3.75
L-Tryptophan	2.50	2.50
L-Tyrosine	4.00	4.00
L-Valine	7.00	7.00
$NaHCO_3$	8.00	3.20
Total weight, g	103.25	50.70

[1] Represents quantities used in the 10% basal diet.
[2] Essential amino acid mixture added to create the imbalance.

to dryness and prepared for running through an amino acid analyzer [2] using the procedure described by Spackman et al. (6) and Moore et al. (7). The effluent fractions corresponding to the labeled histidine or threonine peaks were collected separately and saved for counting to estimate the amounts of intact amino acid incorporated into liver protein.

One-fourth milliliter of blood plasma from each rat was mixed with 5 ml 10% TCA solution and centrifuged. The protein-containing pellet was washed twice with 2.5 ml TCA solution and the TCA fractions were combined and saved. The protein-containing pellet was autoclaved with 5 ml 6 N HCl for 20 hours (121°, 15 lb/in^2), filtered, and saved.

The carcass was treated in the same way for later counting. Also, the upper gastrointestinal tract contents were homogenized, measured, and 5 ml of the homogenates were autoclaved with 5 ml concentrated HCl.

One-gram portions of the different diets were dissolved in 10 ml of 6 N HCl and autoclaved as described before. Carbon-14 analysis of the samples was carried out in a liquid scintillation spectrometer.[3] One-tenth or two-tenths milliliter of the aqueous samples was dispersed in 15 ml of liquid scintillator composed of 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(5-biphenyloxazolyl)]-benzene in a solvent consisting of 20% ethanol in toluene. Dry CO_2 displaced from 1 ml of alkaline solution was trapped in 2 ml Hyamine hydroxide solution and dispersed in 15 ml of liquid scintillator system. Internal standards of

[2] Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
[3] Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

TABLE 2

Diet composition [1]

Ingredient	Basal	Imbalanced	Corrected
	g/kg	*g/kg*	*g/kg*
Basic amino acid mixture [2]	103.25	103.25	103.25
Imbalancing amino acid mixture [2]	—	50.70	50.70
L-Histidine·HCl	—	—	2.5
Vitamin sucrose mixture [3]	50.00	50.00	50.00
Salt mixture [4]	50.00	50.00	50.00
Choline chloride	2.00	2.00	2.00
Corn oil	100.00	100.00	100.00
Alpha-tocopherol	0.23	0.23	0.23
Oleum percomorphum	0.50	0.50	0.50
Sucrose	694.02	643.32	640.82

[1] Each gram provides about 3 μCi of histidine U-^{14}C or threonine U-^{14}C.
[2] See table 1 for composition.
[3] This mixture provided the following quantities of vitamins: (in milligrams) folic acid, 2.0; vitamin B_{12}, 0.05; niacin, 29.0; calcium pantothenate, 35.0; thiamine·HCl, 7.0; riboflavin, 7.0; pyridoxine·HCl, 14.0; biotin, 0.6; menadione, 6.0; and myo-inositol, 1000.0.
[4] Salt mixture of Salmon, W. D. (17).

^{14}C-benzoic acid of known absolute disintegration rate were used to determine the quenching and counting efficiency of each sample.

Total food intake and the amount absorbed were determined for each rat from the data obtained. In addition, excretion, loss and incorporation of the labeled amino acid under investigation into the protein of various tissues and their corresponding acid-soluble and lipid fractions were estimated and compared for the three diet groups and for each of the times of killing.

RESULTS AND DISCUSSION

The presentation will be divided into three parts: A) Histidine-U-^{14}C metabolic studies; B) threonine-U-^{14}C metabolic studies; and C) comparison and general discussion.

The following points are worth mentioning and should be considered throughout the rest of this report: 1) the histidine and threonine experimental diets contain the same isotope concentration (about 3 μCi/g diet); 2) the specific activity of the respective amino acids is different in the three experimental diets. The histidine ratio is 1:1:2 in the basal, histidine-imbalanced and corrected diets, respectively, whereas the threonine ratio is 1:2:2; 3) most of the data are derived from the analysis of the isotope rather than the labeled amino acid per se; 4) each value is the average from two rats per time interval and is expressed as disintegrations per minute (dpm) per gram wet tissue per gram diet absorbed; 5) comparison between imbalanced groups and their respective controls (basal and corrected groups) is always based on the fraction or percentage of the amino acid in the diet that was absorbed and converted to protein or found in the tissue fluids; and 6) estimates of total muscle mass (50% of the body weight) and total blood plasma volume (4.04% of the body weight) (8) were used in deriving the percentage distribution of radioactivity in the different fractions of muscle and blood plasma.

A. Histidine-U-^{14}C metabolic studies. Shown in table 3 are the data describing the total recovery of the absorbed radioactivity from histidine-U-^{14}C and its per-

TABLE 3

Percentage distribution into different tissue fractions and total recovery of radioactivity from histidine U-^{14}C in diets fed to rats

Tissue	Fraction	Basal diet			Imbalanced diet			Corrected diet		
		2 hr	4 hr	6 hr	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
Liver	Protein	11.19	12.23	12.16	12.0	14.3	16.1	10.84	11.3	11.14
	TCA-soluble	7.88	5.55	4.07	6.95	5.16	2.66	10.1	8.13	6.02
	Lipid	0.59	0.73	0.96	0.74	0.73	0.74	0.89	1.20	1.40
Total muscle and remainder of the carcass	Protein	11.2	13.71	18.6	12.94	17.2	21.7	12.64	14.6	16.53
	TCA-soluble	27.8	24.44	21.82	26.33	20.5	15.2	27.79	24.53	23.8
	Lipid	0.49	0.69	0.84	0.56	0.48	0.66	1.12	1.71	1.82
Kidney	Protein	1.35	1.9	2.01	1.49	2.12	2.41	1.16	1.39	1.51
	TCA-soluble	0.94	0.67	0.49	0.81	0.72	0.46	1.01	0.83	0.69
Intestine	Protein	14.88	19.81	18.99	16.03	18.3	20.73	13.04	14.6	13.1
	TCA-soluble	16.43	9.16	5.88	14.3	9.04	4.97	15.16	10.37	6.41
Blood plasma	Protein	3.39	5.65	6.16	4.65	6.39	7.86	3.12	4.72	4.91
	TCA-soluble	0.95	0.8	0.51	0.9	0.7	0.32	1.02	0.85	0.45
CO_2, urine and feces		1.8	3.4	6.0	2.2	3.7	5.1	2.5	6.4	11.4
Total recovery		98.89	98.74	98.49	99.9	99.34	98.91	100.39	100.63	99.19

centage distribution in protein and non-protein fractions of the different tissues, as well as the total losses of radioactivity through expired CO_2, urine and feces. All recovery values were above 98.5%. In experiments of this nature, high recovery values are essential and prerequisite for the validity of the results and their interpretations. The total amounts of radioactivity lost through expired CO_2, urine and feces in addition to those found in the lipid fractions of the imbalanced groups resembled more or less those of the basal control groups, and were less than those of the corrected groups. These results indicated that the overall losses of the limiting amino acid through these channels were not elevated, and did not support the thesis that ingestion of imbalanced diets causes significant catabolic losses of the most limiting amino acid (9). The rest of the data in table 3 and figure 2 portray the general trends observed in all the tissues examined. Higher isotope retention into protein of all tissues of the imbalanced groups was accompanied by a concomitant depression in the isotope concentration of the TCA-soluble fractions. These trends were established as early as 2 hours after the experimental diets were fed.

The set of curves in figure 3 i, ii and iii shows the change with time of radioactivity retained in the different fractions of liver (the most metabolically active tissue), as contrasted to muscle (the least active tissue), and blood plasma which is the transport medium of amino acids from liver to the different body organs. The values were calculated as disintegrations per minute per gram tissue or milliliter blood plasma per gram diet absorbed.

Liver data (fig. 3 i) indicated that the radioactivity level in the lipid fraction of the imbalanced groups was significantly less after 6 hours compared with its respective control groups. On the other hand, radioactivity in the TCA-soluble fractions of the groups receiving the three different diet treatments tended to decrease with time. The values, however, were significantly less for the imbalanced groups even at the 2-hour interval. The incorporation of radioactivity into liver protein

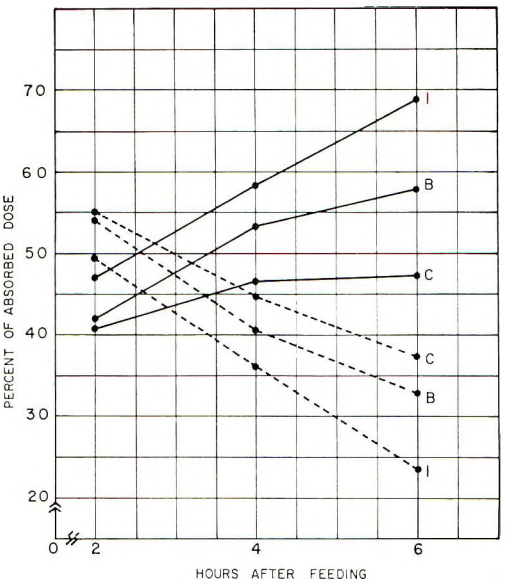

Fig. 2 Percentage of absorbed histidine-U-^{14}C dose retained into all the TCA-protein precipitates (— ● —) and TCA-soluble fractions (. . . ● . . . . .) after feeding basal (B), histidine-imbalanced (I), and corrected (C) diets.

fractions of the imbalanced groups was significantly higher than in their respective control groups at all time intervals. Although radioactivity values indicated that higher ratios of the labeled histidine were retained in liver protein fractions of the imbalanced groups, one must remember that larger amounts of histidine were retained in liver protein fractions of the corrected groups.

Examination of muscle data (fig. 3 ii) showed that ^{14}C incorporation into muscle protein was always higher, favoring the imbalanced groups. Significant differences were demonstrable between the imbalanced and basal controls at the 2- and 4-hour intervals and only after 6 hours when compared with the corrected controls. The TCA-soluble fractions of the imbalanced groups, however, showed significantly lower values of radioactivity after 6 hours. This is in marked contrast to the early significant differences observed in liver. Radioactivity values of the muscle lipid fractions for the imbalanced groups were lower than their respective control groups. Significant depression was only observed,

however, between the imbalanced and corrected groups.

Blood plasma data (fig. 3 iii) indicated that radioactivity values of plasma protein fractions from animals fed the imbalanced diet were significantly higher than their respective controls at all time intervals. The depression in radioactivity content of plasma TCA-soluble fractions of the imbalanced groups was significantly less after 6 hours compared with the basal groups. This significant depression, however, became evident after 4 hours on comparing the imblanced with the corrected groups.

Detailed information about the incorporation data of the kidney and gastrointestinal tract fractions is found in the doctoral thesis.[4] Both tissues showed pronounced effects similar to those of liver.

These results agreed with the previous reports which indicated that enhanced incorporation of the limiting amino acid into protein, with its rapid disappearance from the peripheral circulation and the tissue fluids, are the result of ingesting imbalanced diets (2, 4, 10–13).

Data in table 4 provided additional evidence that the improvement in retention efficiency of the limiting amino acid is accompanied by an increase in the amounts of intact histidine utilized in protein synthesis. The ratio of intact histidine retained into liver protein fractions of the basal and corrected groups corresponded closely to the ratio of histidine found in the respective diets. Furthermore, though the amounts of intact histidine incorporated into liver protein fractions of the control groups were decreasing with time, a reverse trend was evident in the imbalanced groups (i.e., increased incorporation of intact histidine with time). This can be considered additional evidence of improved efficiency of utilization of the most limiting amino acid and enhanced protein synthesis due to the imbalance.

Table 5 projects in detail the isotope losses through expired CO_2, urine and feces. Although these losses were relatively small, interpretation of the trends observed was necessary. This was because some previous reports had exaggerated the role of these losses and considered them as predisposing causes in the adverse effects noticed in the imbalance situations (9, 14). The data indicated that the amounts of $^{14}CO_2$ expired by the imbalanced groups were significantly less than their respective controls after 4 hours. The amounts of radioactivity excreted in urine and feces by the imbalanced groups, however, tended to be higher than their respective basal groups, and significantly lower than the corrected groups. These results disagreed with the report by Florentino and Pearson (9) and supported the conclusions by Wilson et al. (15), Harper and co-workers (2, 13) and Hartman and King (3) that these catabolic losses did not play a decisive role in the imbalance phenomena.

B. Threonine-U-^{14}C metabolic studies. Threonine was selected to study the effect of the histidine imbalance system on the metabolic behavior of nonlimiting indispensable amino acids. This choice was based on the report by Ellison and King (4) that threonine is the second most limiting amino acid in the basal diet. Under this condition, its relative efficiency of utilization should be higher than the rest of the indispensable amino acids. Consequently, any significant deviation in threonine utilization due to the imbalance should strongly support the thesis that normal metabolic behavior of the nonlimiting amino acids is disturbed in animals fed imbalanced diets.

The percentage distribution and recovery of radioactivity in rats fed basal, histidine-imbalanced and corrected diets labeled with threonine-U-^{14}C are shown in table 6. The values were calculated using the same parameters previously followed in histidine metabolic studies. Examination of the data indicated clearly that significant disorders in threonine metabolism were evident. The high recovery values led to no other interpretations except the one already mentioned. Total radioactivity losses through expired CO_2, urine and feces were elevated for the imbalanced groups and reached 10% after 6 hours. This supports the thesis that catabolism

[4] Soliman, A-G. M. 1967 Metabolic derangements in response to ingestion of imbalanced amino acid mixtures. Ph.D. dissertation Virginia Polytechnic Institute, Blacksburg, Virginia.

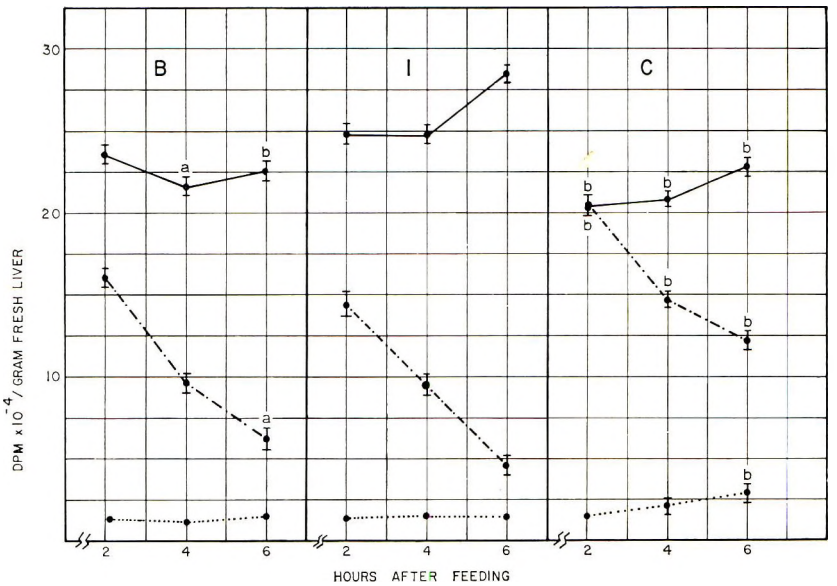

Figure 3-i

Fig. 3 (i-iii) Change with time of radioactivity retained in the different fractions of liver (i), muscle (ii), and blood plasma (iii) after feeding basal (B), histidine-imbalanced (I), and corrected (C) diets labeled with histidine-U-^{14}C. Each value is the average from two rats and calculated as: disintegrations per minute per gram fresh tissue per gram diet absorbed. Protein fraction (——●——); TCA-soluble fraction (—.—●—.—.—); lipid fraction (. . .●. . . . .). a, difference between control and imbalance is statistically significant ($P < 0.05$); b, difference between control and imbalance is statistically significant ($P < 0.01$).

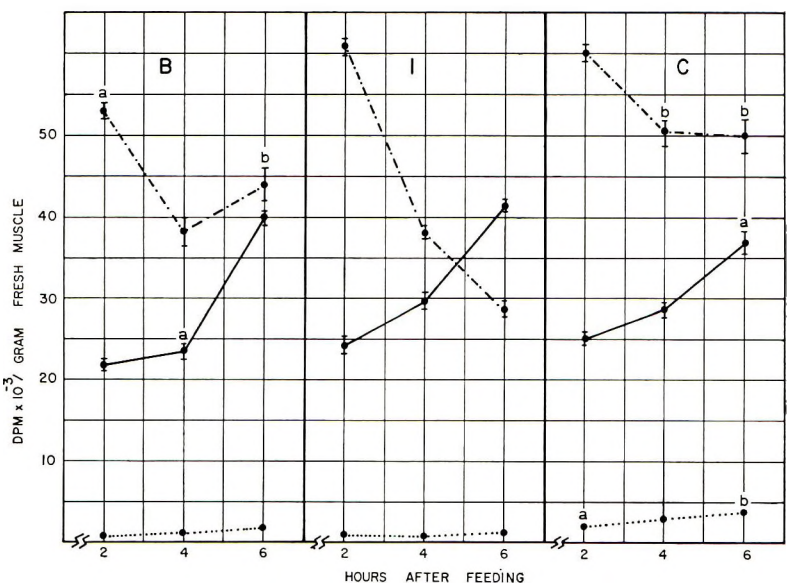

Figure 3-ii

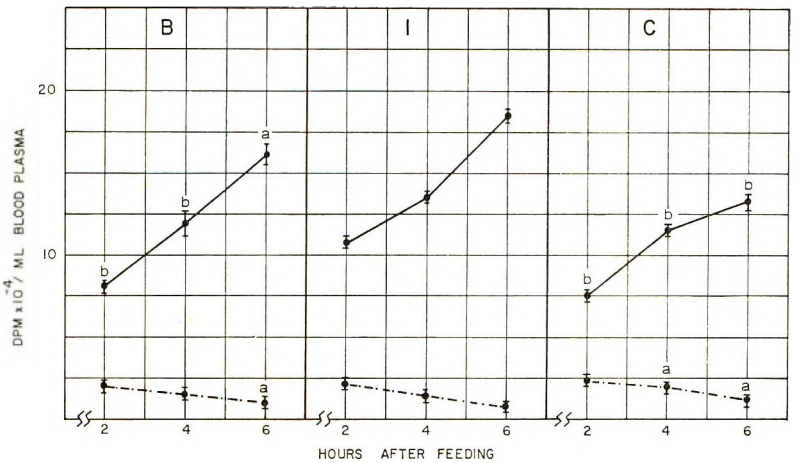

Figure 3-iii

TABLE 4

Micrograms and percentage incorporation of intact histidine-U-^{14}C into liver protein fractions

Diet	Hours after feeding					
	2		4		6	
	% intact [1]	*μg* [2]	*% intact* [1]	*μg* [2]	*% intact* [1]	*μg* [2]
Basal	96.2	83	94.6	75	94.0	76
Imbalanced	97.0	87	94.0	85	92.0	96
Corrected	89.1	131	95.6	144	84.8	141

[1] Percentage of radioactivity found in histidine per 1 g wet tissue weight.
[2] Values represent micrograms incorporated into 1 g wet tissue weight.

TABLE 5

Total radioactivity from histidine-U-^{14}C in expired CO_2, urine and feces [1]

	Time intervals	Basal diet	Imbalanced diet	Corrected diet
	hr			
CO_2	2	82,155 ± 1,800 [a]	147,341 ± 13,789 [2]	66,643 ± 8,954 [a]
	4	142,575 ± 9,790 [a]	115,495 ± 5,270	180,127 ± 7,798 [b]
	6	213,767 ± 20,478 [a]	170,636 ± 1,550	485,876 ± 17,860 [b]
Urine	2	32,519 ± 6,041	47,086 ± 9,534	94,483 ± 2,300 [a]
	4	72,346 ± 4,549	105,795 ± 13,600	188,919 ± 1,490 [a]
	6	94,392 ± 9,880 [b]	180,559 ± 5,800	183,401 ± 9,184
Feces [3]	2	6,298 ± 561 [a]	3,258 ± 535	7,756 ± 460 [a]
	4	17,618 ± 3,500 [a]	35,140 ± 2,012	68,504 ± 4,653 [a]
	6	105,050 ± 10,887	55,636 ± 416	115,584 ± 9,193 [a]

[1] Each value is the average from two rats and calculated as disintegrations per minute resulting from the absorption of 1 g of diet.
[2] Mean ± SEM.
[3] For feces, it must be recognized that the data simply represent the amount of isotope recovered without specifying whether the isotope comes from recycling or from failure to absorb.
[a] Difference between control and imbalance is statistically significant ($P < 0.05$).
[b] Difference between control and imbalance is statistically significant ($P < 0.01$).

of nonlimiting indispensable amino acids is increased in response to ingestion of imbalanced amino acid mixtures. The low percentage of the isotope incorporated into lipid fractions can be explained by the fact that metabolic degradation of threonine does not yield ketogenic bodies as readily as leucine, isoleucine, tyrosine and

TABLE 6

Percentage distribution into different tissue fractions and total recovery of radioactivity from threonine U-^{14}C in diets fed to rats

Tissue	Fraction	Basal diet			Imbalanced diet			Corrected diet		
		2 hr	4 hr	6 hr	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
Liver	Protein	10.74	10.2	12.95	12.27	14.07	11.75	12.38	12.51	13.84
	TCA-soluble	1.27	1.05	0.9	2.45	1.68	1.27	1.84	1.23	1.18
	Lipid	0.98	0.82	1.18	0.49	0.68	0.59	0.53	0.6	0.77
Total muscle and remainder of the carcass	Protein	16.9	26.07	24.21	14.28	16.89	23.46	15.24	20.84	21.95
	TCA-soluble	20.45	18.74	14.72	29.09	26.37	18.94	25.3	20.42	19.13
	Lipid	1.05	2.2	1.89	1.39	1.64	1.29	0.87	1.01	1.26
Kidney	Protein	1.76	1.87	1.96	1.43	1.89	2.02	1.47	1.92	2.13
	TCA-soluble	0.46	0.4	0.32	0.60	0.49	0.4	0.57	0.45	0.39
Intestine	Protein	25.72	22.65	22.4	17.14	18.48	19.5	18.46	20.1	20.88
	TCA-soluble	11.27	7.07	5.47	11.63	5.58	5.07	10.38	7.06	5.14
Blood plasma	Protein	4.68	4.33	7.01	3.78	5.65	5.39	7.86	5.66	5.93
	TCA-soluble	0.63	0.6	0.41	1.46	0.9	0.83	1.39	0.82	0.77
CO_2, urine and feces		3.3	3.9	7.5	3.2	5.2	10.0	4.1	5.8	6.0
Total recovery		99.21	99.9	100.92	99.21	99.52	100.51	100.89	98.42	99.37

phenylalanine. These amino acids are also found in excess in the imbalanced mixture and are catabolized simultaneously. In addition, threonine utilization in the synthesis of other nonessential amino acids may be another reason for the observed low values in the lipid fractions. More evidence supporting this will be provided later.

It is evident from the rest of the various tissue data (table 6) and the retention values (fig. 4) that utilization of threonine in protein synthesis was lowered and that the levels of radioactivity in the TCA-soluble fractions were altered and showed significantly higher values in response to the imbalance.

Figures 5 i, ii and iii provided detailed information about the average distribution ratios of radioactivity in the different fractions of liver, muscle and blood plasma at different time intervals after feeding the experimental diets labeled with threonine-U-^{14}C. It is evident that radioactivity levels in TCA-soluble fractions of all the tissues examined were significantly higher for the imbalanced groups. Except for liver, pro-

Fig. 4 Percentage of absorbed threonine-U-^{14}C dose retained into all the TCA-protein precipitates (——●——) and TCA-soluble fractions (. . . ● . . . . .) after feeding basal (B), histidine-imbalanced (I), and corrected (C) diets.

tein fractions of muscle and blood plasma of the imbalanced groups showed lower incorporation values when compared with their respective controls. On the other hand, the metabolically active tissues behaved differently. Liver protein fractions of the imbalanced groups showed higher incorporation values at the three time intervals on comparison with the respective control groups. However, from the kidney and intestinal data (found in the doctoral thesis) [5] the incorporation values into protein fractions of the former tissue were higher than the basal groups whereas the latter tissue resembled muscle in its behavior. If one considers the actual counts as presented in figures 5 i, ii and iii, together with threonine concentration in the imbalanced diet, it becomes clear that higher net retention of threonine occurred while its efficiency of utilization was decreasing. Additional support for this statement is evident from the data in table 7 which show the percentage incorporation of intact threonine in liver protein fractions. Despite the fact that the amounts of intact threonine were still higher in the groups receiving imbalanced diet, the percentage ratio of intact threonine incorporated into the protein fractions was very low and reached about 50% after 6 hours. In addition, these results clearly indicated that threonine was used in the synthesis of other deficient amino acids, namely, the dispensable ones. Furthermore, the elevation in the amounts of threonine incorporated into liver protein fractions of the imbalanced groups (table 7), and the corresponding elevation noticed before in the amounts of histidine (table 4) provided clear-cut evidence of enhanced protein synthesis due to ingestion of imbalanced diets.

Table 8 summarizes the radioactivity losses through expired CO_2, urine and feces. Contrary to the observations noted in histidine studies, radioactivity from threonine in CO_2 expired by the imbalanced groups tended to increase with time and were significantly higher than their respective controls. Urine and fecal data indicated that there were no significant differences between the imbalanced groups and their respective controls, and that these losses were proportional to threonine content in the diets.

C. Comparison and general discussion. Since elucidation of the metabolic derangements in response to ingestion of imbalanced mixtures of amino acids was an important aspect of this investigation, due attention was paid to the controls used in these studies. Differences in experimental design between this set of experiments and those of Harper and coworkers (2, 13) and Hartman and King (3) lie in the nature of the controls. In the present investigation the two control diets were combined. Control diet 1 (basal diet) contained the same histidine level, whereas control diet 2 (corrected diet) had almost the same protein content as the imblanced diet but supplemented with histidine to correct the imbalance. Under these tight restrictions, any significant difference observed in the metabolic behavior of the limiting amino acid could be attributed only to the imbalance. Studying the metabolic behavior of threonine under the same conditions constitutes another control.

At this point, it is worthy to discuss the data in table 9 which provided further support to the thesis that altered free amino acid patterns of plasma and tissue fluids are due to the metabolic derangements that occurred after intestinal absorption. The data confirm the earlier findings (1–3, 13, 16) that absorption is not hampered as a result of ingesting imbalanced amino acid mixtures. The values in the table indicated the percentage of radioactivity absorbed at different time intervals after feeding histidine-U-^{14}C and threonine-U-^{14}C diets. Absorption was calculated as the difference between the amounts of radioactivity ingested and those recovered from the gastrointestinal tract. Differences between imbalanced and control groups were statistically insignificant.

From the previous discussion of histidine and threonine metabolic studies it is evident that ingesting an imbalanced diet resulted in increased net retention of histidine (the most limiting amino acid) and a corresponding increase in the amounts of threonine retained. This provided clear

[5] See footnote 4.

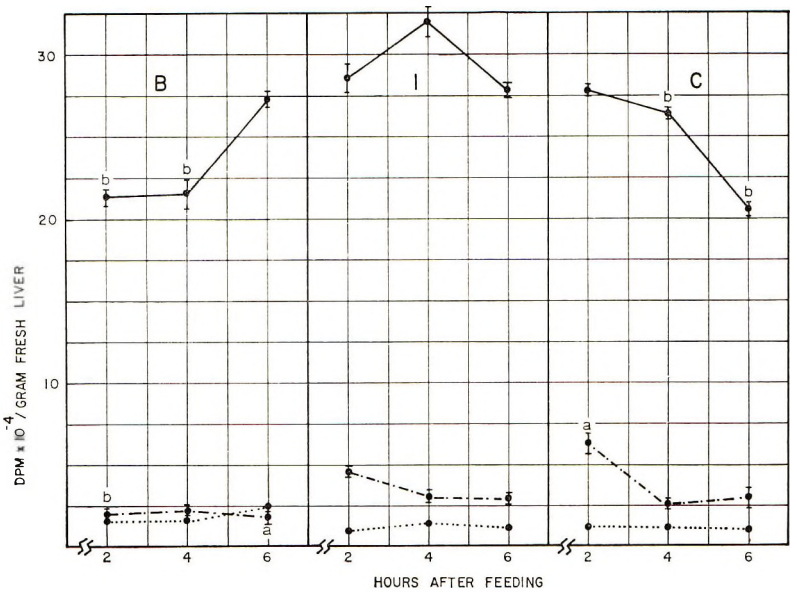

Figure 5-i

Fig. 5 (i-iii) Change with time of radioactivity retained in the different fractions of liver (i), muscle (ii), and blood plasma (iii) after feeding basal (B), histidine-imbalanced (I), and corrected (C) diets labeled with threonine-U-^{14}C. Each value is the average from two rats and calculated as: distintegrations per minute per gram fresh tissue per gram diet absorbed. Protein fraction (——●——); TCA-soluble fraction (—.—●.—.—); lipid fraction (. . . ● . . . . .). a, difference between control and imbalance is statistically significant ($P < 0.05$); b, difference between control and imbalance is statistically significant ($P < 0.01$).

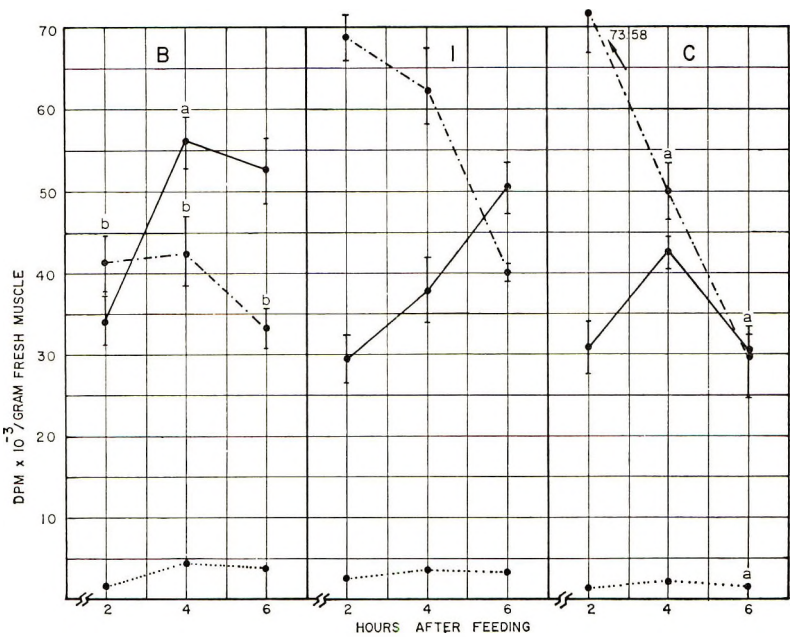

Figure 5-ii

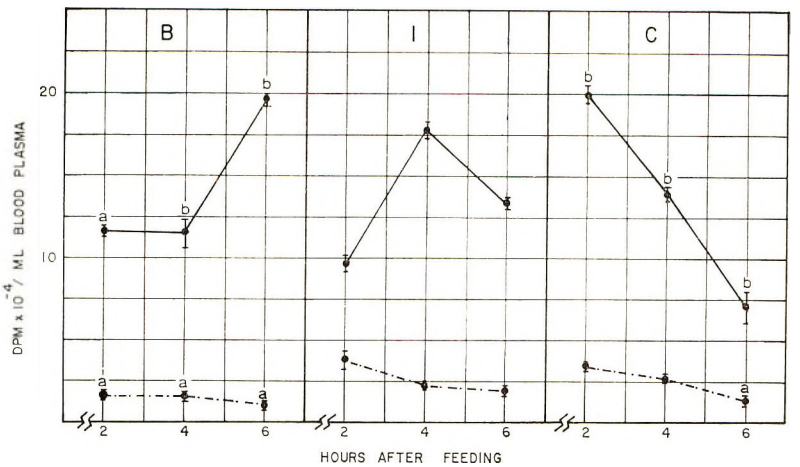

Figure 5-iii

TABLE 7

Micrograms and percentage incorporation of intact threonine-U-^{14}C into liver protein fractions

Diet	Hours after feeding					
	2		4		6	
	% intact [1]	*μg* [2]	*% intact* [1]	*μg* [2]	*% intact* [1]	*μg* [2]
Basal	91.5	107	86.9	102	78.0	116
Imbalanced	60	180	48.0	160	52.3	152
Corrected	81.3	247	67.3	193	76.5	172

[1] Percentage of radioactivity found in threonine per 1 g wet tissue weight.
[2] Values represent micrograms incorporated into 1 g wet tissue weight.

TABLE 8

Total radioactivity from threonine-U-^{14}C in expired CO_2, urine and feces [1]

	Time intervals	Basal diet	Imbalanced diet	Corrected diet
	hr			
CO_2	2	45,276 ± 1,037 [b]	26,576 ± 4,306 [2]	39,906 ± 9,250
	4	51,512 ± 4,926 [b]	125,001 ± 3,358	92,506 ± 1,475 [b]
	6	169,061 ± 3,278 [b]	246,025 ± 4,289	205,255 ± 5,527
Urine	2	6,128 ± 1,250	5,979 ± 574	7,773 ± 528
	4	18,410 ± 4,512	11,919 ± 2,028	13,599 ± 345
	6	20,495 ± 9,580	25,943 ± 2,800	19,559 ± 3,630
Feces	2	6,559 ± 1,196	6,379 ± 715	8,299 ± 1,675
	4	10,594 ± 888	14,175 ± 2,901	32,744 ± 7,649
	6	47,995 ± 2,252 [a]	58,641 ± 3,505	43,112 ± 8,223 [a]

[1] Each value is the average from two rats and calculated as disintegrations per minute resulting from the absorption of 1 g of diet.
[2] Mean ± SEM.
[a] Difference between control and imbalance is statistically significant ($P < 0.05$).
[b] Difference between control and imbalance is statistically significant ($P < 0.01$).

evidence of enhanced protein synthesis. The data in table 10, however, describe the retention efficiency of both amino acids. Retention efficiency of histidine was higher in the imbalanced group after 6 hours (94.63%) compared with the basal and corrected control groups (93.91 and 88.51%, respectively). The opposite be-

TABLE 9

Percentage of absorbed radioactivity after feeding histidine U-^{14}C or threonine U-^{14}C diets [1]

Diet	Hours after feeding		
	2	4	6
Basal	41.3	68.9	86.5
Imbalanced	45.8	68.5	83.1
Corrected	41.0	52.5	90.2

[1] Each value is the average from four rats (two receiving histidine diet and two receiving threonine diet) calculated as percentage absorption = $\frac{\text{total radioactivity absorbed}}{\text{radioactivity ingested}} \times 100$.

havior was apparent with threonine. After 6 hours the retention efficiency was 90.04% for the imbalanced group which was lower than the corresponding control groups (about 93%).

Incorporation data in the TCA-soluble fractions of the different tissues examined indicated that the levels of the free limiting amino acid (histidine) in plasma and tissue fluids of the imbalanced groups were severely depressed, while levels of the nonlimiting indispensable amino acid (threonine) were elevated. This leads to the only conclusion, that the efficiency of utilization of the most limiting amino acid in protein synthesis is increased while that of the nonlimiting indispensable amino acid is decreased as a result of ingesting imbalanced amino acid mixture. Positive support for this conclusion is provided by the data in table 11. The efficiency-of-utilization values were calculated as the percentage ratio of radioactivity retained in the total protein fractions to that absorbed. Variance analysis indicated that all the values of the imbalanced groups were significantly higher for histidine whereas threonine values were significantly lower than their respective basal and corrected control groups.

Since more studies were conducted on liver, this tissue will be used, together with the information about diet composition found in tables 1 and 2, to explain the high incorporation values of threonine and the differences in the efficiency of utilization observed with both amino acids. If one considers the concentration of the two amino acids in the different diet treatments, it is obvious that histidine is the first limiting amino acid in the three diets. However, amino acid proportions in the basal diet are relatively best adjusted to

TABLE 10

Retention efficiency of histidine and threonine in the whole animal [1]

Diet	Histidine U-^{14}C			Threonine U-^{14}C		
	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
Basal	98.18	96.51	93.91	96.66	96.09	92.54
Imbalanced	97.79	96.23	94.63	96.76	94.75	90.04
Corrected	97.51	93.61	88.51	95.94	94.08	93.97

[1] Each value is the average from two rats and is expressed as percentage of the total radioactivity absorbed and recovered. Retention efficiency = $\frac{\text{total radioactivity in all the different tissue fractions}}{\text{total radioactivity recovered}} \times 100$.

TABLE 11

Efficiency of utilization (EU) of histidine and threonine into protein [1]

Diet	Histidine U-^{14}C			Threonine U-^{14}C		
	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
Basal	42.0	53.3	57.9	59.8	65.1	68.5
Imbalanced	47.1	58.3	68.8	48.9	57.0	62.1
Corrected	40.8	46.6	47.2	55.4	61.0	64.7

[1] Each value is the average from two rats and expressed as percentage of the total radioactivity absorbed. EU = $\frac{\text{radioactivity in the total protein}}{\text{radioactivity absorbed}} \times 100$.

meet the requirements for protein synthesis. In the corrected diet, histidine is still the apparent first limiting amino acid; another major limiting factor, but not immediately evident, is the very low content of the dispensable amino acids. Consequently, some catabolic and synthetic processes must take place to provide the adequate amino acid proportions. Surplus amounts of indispensable amino acids are used in these processes. The net result is low efficiency of utilization of the most limiting amino acid (histidine) and still lower depression in the efficiency of utilization of the other nonlimiting indispensable amino acids, as demonstrated by threonine in the corrected groups. In the case of the imbalanced diet, the interpretation is still valid with respect to the surplus indispensable amino acids. This may account for the almost doubled incorporation values of threonine into the protein fractions of the different tissues of rats fed the imbalanced diet. However, the behavior of the limiting amino acid is completely different. Instead of the expected low efficiency there was a significant increase in its efficiency of utilization and the amounts of intact amino acid incorporated into protein. This explanation is supported by the data in tables 4 and 7, and the similar observations reported for tryptophan imbalances when studied with labeled tryptophan (15).

It should be pointed out here that the results obtained using histidine-U-^{14}C agree with and complement the previous ^{15}N-isotope studies reported from this laboratory (3). This agreement indicates that the conclusions drawn from the isotope studies are valid and reflect the actual behavior of the intact amino acid (limiting amino acid), and not an artifact due to the utilization and incorporation of the labeled degradation products from this amino acid.

At this point we feel the necessity to discuss an alternate interpretation of our results. The data may suggest that the increased rate of histidine or threonine incorporation into protein due to imbalance may reflect a change in rate of protein synthesis, or a change in the specific activity of the particular tissue-free amino acid pool. This could be the case if histidine data provided in this report are considered alone. However, our conclusion that protein synthesis is enhanced due to feeding an imbalanced diet is based on our results, and those found in the previous reports (3, 4, 11, 13, 18) in which the histidine-imbalance system was studied. All reports indicate that histidine concentration and isotope levels from labeled histidine in free amino acid pools follow the same pattern. This was evident when acid-soluble fractions of various tissues and plasma of rats receiving the imbalanced and control diets were examined. Accordingly, we do not believe that the difference between specific activity of free histidine in tissue pools of rats receiving the imbalanced and control diet reaches such a magnitude that it allows the interpretation of our results in both ways.

Further support to the conclusion that protein synthesis is enhanced subsequent to ingestion of imbalanced diets is provided by our threonine studies and by the report of Sanahuja and Harper (18). This report showed that plasma levels of threonine were substantially elevated in rats receiving a histidine-imbalanced diet. The same behavior was demonstrated when radioactive threonine was used in our studies. Since the trends of amino acid levels observed in plasma are reflections of the trends in the free amino acid pools of various tissues, one can conclude that specific activity of free threonine will be lower in rats receiving histidine-imbalanced diets labeled with radioactive threonine. Consequently, one expects the amounts of labeled threonine incorporated into tissue proteins of rats receiving the imbalanced diet to be lower than their respective controls. The opposite behavior was evident from threonine data of liver protein fractions. In addition, the concurrent increase in the amounts of labeled histidine and threonine incorporated into proteins (although the levels of histidine in tissue pools are low while the levels of threonine are high) is considered as sufficient evidence of enhanced protein synthesis.

So far these results confirm the hypothesis advanced by Harper and co-workers

(1, 2) that ingestion of imbalanced amino acid mixtures results in severe depression in the levels of the limiting amino acid in plasma and tissue fluids. This is severely aggravated by the increased incorporation and utilization of the limiting amino acid in protein. Consequently, the probability of subjecting the limiting amino acid to the action of the degrading enzymes is decreased. This is evidenced by the significant depression in the limiting amino acid catabolic products in the imbalance situation. Furthermore, these results agree with previous reports (2, 3, 13) and show that retention efficiency and efficiency of utilization of the limiting amino acid are elevated, and that protein synthesis is enhanced as a result of ingesting imbalanced amino acid mixtures. In addition, the data confirm the assumption that nonlimiting indispensable amino acids behave differently—their retention efficiency and efficiency of utilization are lowered despite the fact that there is an increase in their net retention. The fact that these metabolic derangements occurred at very early times, while the food was still being absorbed from the intestinal lumen, renders its support to the hypothesis that these disorders are the initial effects, and their metabolic consequences trigger an appetite-controlling mechanism which results in depressed food intake and retarded growth.

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Effect of Dietary Magnesium and Fluoride on Bone Mineralization in Young Chicks [1,2,3]

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ABSTRACT Tracer techniques were used to examine the reduced bone mineralization observed in young chicks fed diets elevated in both magnesium (4500 ppm) and fluoride (800 ppm). The distribution of ^{45}Ca 1 hour after injection of the isotope into a ligated intestinal segment was examined in 2-day-old chicks fed control and high magnesium plus high fluoride diets. Although no differences in intestinal absorption were observed, decreased femur uptake and increased plasma levels of ^{45}Ca were found in the chicks fed the high magnesium plus high fluoride diets. At an early age (2 to 6 days) chicks fed the high magnesium plus high fluoride diets incorporated less ^{45}Ca and ^{32}P into their femurs 4 hours after intraperitoneal injection when compared with chicks maintained on control, high fluoride or high magnesium diets. As a result of the reduced femur ^{45}Ca incorporation, plasma ^{45}Ca levels were elevated in the high magnesium plus high fluoride group. At later ages (8 to 10 days) incorporation of injected ^{32}P into femurs was not affected by dietary treatment, but ^{45}Ca incorporation was elevated in the high magnesium plus high fluoride group. Analysis of plasma and soft tissues for calcium revealed no treatment differences at 4 days, but ^{45}Ca levels in plasma 4 hours after intraperitoneal injection were elevated above control values at every age studied. Results of in vitro studies have suggested that bone from chicks in the magnesium plus fluoride group incorporate less ^{45}Ca and ^{32}P than bones from chicks in the other groups when incubated in plasma of chicks from the same treatment.

Previous work has demonstrated that a reduction in growth rate and bone mineralization resulted from feeding chicks diets containing elevated levels of magnesium and fluoride (1, 2). Gardiner et al. (1) reported that after 4 weeks on a high magnesium plus high fluoride diet chicks displayed a characteristic leg weakness in addition to decreased body weight and bone calcium and phosphorus levels compared with chicks fed control, high fluoride or high magnesium diets. Further study by Griffith et al. (2) revealed that the magnesium × fluoride inhibitory effects could be decreased but not eliminated by increasing the calcium content of the diets from 1.0 to 1.5%. Elevation of the dietary phosphorus level from 0.7 to 1.0% was without effect in alleviating the detrimental effects of the high magnesium plus high fluoride diet (1). Using a practical diet Griffith et al. (3) demonstrated an increase in both total and diffusible plasma magnesium levels as a result of high magnesium treatment alone, but a further increase in total and diffusible plasma magnesium levels was obtained by fluoride supplementation of the high magnesium diet.

The experiments reported here were designed to study further the effects of dietary magnesium and fluoride treatment on calcium and phosphorus metabolism. With the aid of radiocalcium and radiophosphorus, intestinal transport, bone incorporation and in vitro bone uptake experiments were performed.

EXPERIMENTAL

Animals and diets. One-day-old Hubbard White Mountain chicks were randomly assigned to four groups; they were banded and placed in electrically heated, wire-floored batteries. The semipurified

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[2] Some of these data were presented at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1968, Federation Proc., 27: 312 (abstract).

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basal diet [5] was found by analysis to contain 0.058% Mg and 0.0001% F. Magnesium carbonate and sodium fluoride were used to prepare diets containing 0.08% supplemental fluoride (high fluoride), 0.45% supplemental magnesium (high magnesium) and 0.45% supplemental magnesium plus 0.08% supplemental fluoride (high magnesium plus high fluoride). The four diets and deionized water were supplied ad libitum.

In vivo isotope experiments. The metabolism of both ^{45}Ca and ^{32}P was studied in chicks which had been fed each of the four diets for periods ranging from 2 to 10 days. One-half milliliter of an isotope solution containing 1 μCi/ml of ^{45}Ca and/or $^{32}P_i$ dissolved in 0.9% NaCl was administered intraperitoneally to the chicks. At various times thereafter, blood was drawn by cardiac puncture using heparin as the anticoagulant, and plasma was obtained after centrifugation. Immediately thereafter the animals were killed by cervical dislocation; the right femur was removed, freed of adhering tissue and weighed. In one experiment, samples of liver, heart and skeletal muscle were also removed, weighed and analyzed for total calcium, ^{45}Ca and ^{32}P.

Intestinal transport of ^{45}Ca. To examine the effect of dietary magnesium plus fluoride treatment on intestinal transport in 2-day-old chicks, an experiment similar to that described by Wasserman (4) was performed. Chicks were anesthetized by an intramuscular injection of pentobarbital (30 mg/kg body weight). The abdominal cavity was opened and a segment of the duodenum ligated (3.5 cm). One-tenth milliliter of an isotonic saline solution containing 1.27 μCi of ^{45}Ca and 1500 ppm of carrier Ca was injected into the ligated segment which was then placed back into the body cavity and the incision sutured with surgical clamps. One hour later blood was removed by cardiac puncture and the animal killed. The ^{45}Ca content of plasma, femur and the ligated intestinal section was determined.

In vitro uptake experiments. The incorporation of ^{45}Ca and ^{32}P into bone fragments was examined in vitro by incubating femurs with plasma in the presence of the radioisotopes. Blood from chicks which had been fed each of the four diets for 2 days was taken by cardiac puncture with a heparinized needle and syringe and placed in ice-cold centrifuge tubes. Following centrifugation of the blood in a refrigerated centrifuge [6] the pooled plasma from each of the four groups was transferred to a chilled beaker. Five 2-ml aliquots of each of the pooled plasma samples were added to 25-ml chilled Erlenmeyer flasks containing 100 μl of an isotonic saline solution containing 1 μCi each of ^{45}Ca and ^{32}P. To these flasks were added the fragments of a weighed femur which had been cut cross-wise in the middle and each end split longitudinally with a razor blade. The femurs were incubated with plasma obtained from the same group in a Dubnoff metabolic shaker [7] at 41°. After 2 hours, the femur fragments were removed, washed three times with deionized water and analyzed for ^{45}Ca and ^{32}P.

Analyses. One milliliter of plasma was added to 10 ml of a solution containing 4.4% trichloroacetic acid (TCA) and 1.1% La. After thorough shaking the precipitated protein was removed by centrifugation. One milliliter of the TCA supernate was added to 15 ml of scintillator solution (5) and counted in a liquid scintillation counter.[8] Counts were taken in two channels, and simultaneous equations as described by Okita et al. (6) were employed to determine the amount of ^{45}Ca and ^{32}P in the sample. Another 1-ml aliquot of the TCA supernate was taken and inorganic phosphate determined by the method of Allen (7). Calcium determination was made directly on the remaining TCA supernate by atomic absorption spectrophotometry.[9]

Femurs and soft tissues were ashed overnight in a muffle oven at 600°. The ash was dissolved in 3 ml of 1 N HCl, and

[5] The percentage composition of the basal diet was: isolated soy protein, 30; corn oil, 5; cellulose, 3; DL-methionine, 0.50; glycine, 0.30; butylated hydroxytoluene (25%), 0.05; vitamin premix, 1.26; mineral mix, 4.41; glucose, as necessary to total 100%. The composition of the vitamin and mineral mixes is given in reference 2.

[6] Ivan Sorvall Inc, Norwalk, Conn.

[7] Precision Scientific Company, Chicago, Ill.

[8] Model 314 EV, Packard Instrument Company, Downers Grove, Ill.

[9] Model 214, Perkin-Elmer Corporation, Norwalk, Conn.

aliquots were taken for ^{45}Ca, ^{32}P and total calcium and phosphorus analyses by methods described above.

Standard errors (SE) were calculated for all means and Student's *t* test was applied to determine significance of differences between means.

RESULTS

The effect of supplemental magnesium and fluoride on growth rate and the development of the characteristic leg weakness was similar to that previously reported (1–3). Supplemental magnesium alone did not alter growth rate; supplemental fluoride reduced growth rate slightly but the growth rate of chicks fed the high magnesium plus high fluoride diet was greatly reduced.

The effect of age and dietary treatment on femur calcium and phosphorus levels is illustrated in table 1. High dietary fluoride appeared to produce no anomalous effects on femur calcium or phosphorus content at any of the times studied. High magnesium treatment resulted in decreased femur calcium levels at 1 and 2 days; however, the greatest reduction was observed in the high magnesium plus high fluoride group at 2 and 7 days. In the combination group (high magnesium plus high fluoride) femur calcium and phosphorus levels were lower than in the other three groups at both 2 and 7 days.

The effect of dietary treatment on femur incorporation of injected ^{45}Ca and ^{32}P is shown in table 2. In the first part of the table, a dramatic reduction in isotope incorporation can be observed in the combination group at 4 and 48 hours after injection. The data in the second part of table 2 show that a reduction in ^{45}Ca and ^{32}P incorporation by the high magnesium plus high fluoride treatment is still apparent 6 days after injection.

The possibility that high magnesium plus high fluoride treatment may produce its effect on bone mineralization by inhibiting intestinal transport of calcium was rejected on the basis of the data shown in table 3. Although decreased femur incorporation of ^{45}Ca and increased plasma ^{45}Ca levels were observed in the combination group, the absorption of ^{45}Ca was not reduced in the magnesium plus fluoride group.

Presented in table 4 is the calcium content of several soft tissues, plasma and femurs as affected by dietary treatment. No significant group differences were observed except for plasma and femur calcium levels which were reduced in both the magnesium and magnesium plus fluoride groups, but the extent of the femur calcium reduction was considerably greater in the combination group. These data clearly show that the magnesium × fluoride inhibitory effect is specific for bone.

The effects of age and dietary treatment on ^{45}Ca and ^{32}P incorporation into femurs are illustrated in tables 5 and 6. Consistent with the results in table 2, the data

TABLE 1

Effect of age and dietary treatment on femur calcium and phosphorus levels [1]

Element	Treatment			
	Control	High fluoride	High magnesium	High magnesium + high fluoride
		1 day		
Calcium	3.41 ± 0.20	3.10 ± 0.04	2.75 ± 0.22	3.00 ± 0.07
Phosphorus	1.87 ± 0.11	1.73 ± 0.05	1.63 ± 0.14	1.67 ± 0.07
		2 days		
Calcium	3.81 ± 0.06	3.81 ± 0.12	3.30 ± 0.09 **	2.79 ± 0.11 **
Phosphorus	1.50 ± 0.03	1.72 ± 0.11	1.49 ± 0.10	1.31 ± 0.05 *
		7 days		
Calcium	4.63 ± 0.13	4.55 ± 0.52	4.75 ± 0.31	3.36 ± 0.36 *
Phosphorus	2.41 ± 0.07	2.24 ± 0.16	2.17 ± 0.16	1.69 ± 0.17 **

[1] Data are expressed as milligrams element per 100 mg fresh bone weight. Average of 5 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

TABLE 2

Femur ^{45}Ca and ^{32}P levels at various times after intraperitoneal injection of ^{45}Ca and ^{32}P in 2-day-old chicks

Time post-injection	Treatment			
	Control	High fluoride	High magnesium	High magnesium + high fluoride
		^{45}Ca (% dose/femur)		
4 hours	3.86 ± 0.12 [1]	4.08 ± 0.14	3.71 ± 0.14	2.85 ± 0.16 **
48 hours	4.97 ± 0.17	5.01 ± 0.14	4.73 ± 0.09	3.67 ± 0.44 *
		^{45}Ca (% dose/femur)		
4 hours	3.40 ± 0.10	3.63 ± 0.12	3.34 ± 0.18	2.36 ± 0.24 **
6 days	3.31 ± 0.28	3.58 ± 0.17	2.72 ± 0.18	2.39 ± 0.15 *
		^{32}P (% dose/femur)		
4 hours	1.65 ± 0.07	1.79 ± 0.08	1.70 ± 0.12	1.19 ± 0.07 **
6 days	3.33 ± 0.08	3.70 ± 0.12 *	3.07 ± 0.10	2.04 ± 0.30 **

[1] Average of 5 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

TABLE 3

Effect of dietary magnesium and fluoride treatment on the intestinal transport and distribution of ^{45}Ca [1]

Treatment	Intestine	Femur	Plasma
	% absorbed [2]	*% of absorbed dose/femur*	*% of absorbed dose/ml*
Control	38 ± 5 [3]	3.62 ± 0.30	1.01 ± 0.13
High magnesium + high fluoride	46 ± 8	2.48 ± 0.29	1.43 ± 0.08 *

[1] Calcium-45, 1.27 μCi, was injected into a ligated section of the duodenum of chicks which had been fed the diets for 2 days. Analyses were performed 1 hour after injection.

[2] Percentage absorbed $= \frac{(\text{total cpm } ^{45}Ca \text{ injected into ligated section} - \text{cpm } ^{45}Ca \text{ remaining after 1 hour uptake})}{\text{total cpm } ^{45}Ca \text{ injected into ligated section}} \times 100.$

[3] Average of 7 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.

TABLE 4

Effect of dietary magnesium and fluoride on calcium content of several tissues in the 4-day-old chick

Tissue [1]	Treatment			
	Control	High fluoride	High magnesium	High magnesium + high fluoride
Liver, ppm	70 ± 5 [2]	70 ± 5	62 ± 3	77 ± 10
Heart, ppm	62 ± 3	71 ± 7	52 ± 5	66 ± 3
Muscle, ppm	54 ± 4	57 ± 4	64 ± 6	55 ± 3
Plasma, ppm	110 ± 1	108 ± 2	102 ± 3 *	103 ± 1 **
Femur, %	3.68 ± 0.07	3.80 ± 0.06	2.87 ± 0.07 **	2.16 ± 0.06 **

[1] Calculated on the basis of fresh weight.
[2] Average of 15 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

in table 5 show that dietary magnesium plus fluoride treatment inhibited the incorporation of both ^{45}Ca and ^{32}P into the femurs of 2-day-old chicks. Different effects, however, were observed with the 7-day-old chicks. No significant treatment effects on ^{32}P incorporation were observed at 7 days, and an actual increase in ^{45}Ca incorporation occurred in the chicks fed the dietary magnesium plus fluoride for 7

TABLE 5

Effect of age and dietary treatment on the incorporation of ^{45}Ca and ^{32}P into femurs 4 hours after intraperitoneal injection

Days on treatment	Treatment			
	Control	High fluoride	High magnesium	High magnesium + high fluoride
	^{45}Ca (% dose/femur)			
2	3.70 ± 0.12 [1]	3.52 ± 0.11	3.27 ± 0.17	2.36 ± 0.11 **
7	4.90 ± 0.08	4.83 ± 0.13	4.89 ± 0.09	6.18 ± 0.42 *
	^{32}P (% dose/femur)			
2	1.86 ± 0.47	1.67 ± 0.12	1.68 ± 0.05	1.01 ± 0.04
7	3.05 ± 0.05	2.74 ± 0.11 *	3.54 ± 0.48	3.10 ± 0.19

[1] Average of 5 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

days. A similar and more detailed study is displayed in table 6. When compared with the other three groups, the high magnesium plus high fluoride treatment resulted in decreased femur calcium and phosphorus levels at every day studied beyond day 2. Femur incorporation of ^{45}Ca was reduced at 2 days by the combination treatment and at 4 days by both the high magnesium and the high magnesium plus high fluoride treatments. At 8 and 10 days, however, ^{45}Ca incorporation into femurs was higher in the combination group. Although these differences (table 6) were not statistically significant, significant differences were observed in other trials not reported, and the difference at 7 days was statistically significant in the trial reported in table 5. The incorporation of ^{32}P was reduced by the high magnesium plus high fluoride treatment at 4 and 6 days and was not elevated at the older ages. Although femur and body weight differences may influence the incorporation of the isotopes, the same relative differences were observed when corrections were made for differences in body and femur weights.

In table 7 the effect of age and diet on plasma calcium metabolism is summarized. The most striking observation was the three-fold elevation in plasma ^{45}Ca levels in the combination group at every age studied. This phenomenon was further studied, and the results are presented in figure 1. Here it is seen that the plasma ^{45}Ca levels remain elevated in the combination group over the 2-day period studied.

The results of an experiment designed to examine the effect of dietary treatment on ^{45}Ca and ^{32}P uptake by femur slices in vitro are presented in table 8. After a 2-hour incubation period, there does appear to be decreased isotope incorporation in the high magnesium plus high fluoride group.

DISCUSSION

In accord with the in vitro results obtained by Goldenberg and Sobel (8), the experiments reported here demonstrate inhibition of bone mineralization by dietary fluoride in the presence of high levels of dietary magnesium. The results of the isotope studies are interpreted as follows. In the combination group, mineral deposition into femurs is inhibited at an early age. This inhibition is demonstrated by a decreased femur uptake of injected ^{45}Ca and ^{32}P. Because of the reduction in femur ^{45}Ca, plasma ^{45}Ca levels become elevated in the magnesium plus fluoride group. A similar reduction in incorporation of injected ^{45}Ca into bone and an increase in plasma ^{45}Ca levels have been observed in hypophysectomized animals (9). After treatment for 6 to 8 days, the inhibitory effect of the high magnesium plus high fluoride treatment subsides.

The consistent observation of an increased incorporation of injected ^{45}Ca into bones of chicks fed the high magnesium plus high fluoride diet for 7 to 10 days may have been caused by some compensatory mechanism which allows the poorly mineralized bones to incorporate the injected

TABLE 6

Effect of age and dietary treatment on calcium and phosphorus content and ^{45}Ca and ^{32}P incorporation into femurs 4 hours after intraperitoneal injection

Treatment	Ca	^{45}Ca	P	^{32}P
	mg/100 mg fresh bone	*% dose/femur*	*mg/100 mg fresh bone*	*% dose/femur*
		2 days		
Control	3.23 ± 0.11 [1]	3.75 ± 0.08	1.80 ± 0.30	1.75 ± 0.06
High fluoride	3.37 ± 0.13	3.85 ± 0.14	1.49 ± 0.11	1.71 ± 0.06
High magnesium	3.34 ± 0.11	3.48 ± 0.20	1.51 ± 0.08	1.75 ± 0.10
High magnesium + high fluoride	3.24 ± 0.18	2.44 ± 0.47 *	1.64 ± 0.19	1.43 ± 0.30
		4 days		
Control	4.04 ± 0.09	4.58 ± 0.15	1.99 ± 0.03	2.50 ± 0.08
High fluoride	3.71 ± 0.25	4.39 ± 0.08	1.94 ± 0.07	2.38 ± 0.08
High magnesium	3.56 ± 0.13 *	3.92 ± 0.21 *	2.00 ± 0.14	2.25 ± 0.27
High magnesium + high fluoride	2.44 ± 0.08 **	3.89 ± 0.34	1.50 ± 0.14 *	1.59 ± 0.24 **
		6 days		
Control	5.15 ± 0.33	4.67 ± 0.06	2.26 ± 0.14	2.73 ± 0.09
High fluoride	4.97 ± 0.19	4.55 ± 0.12	2.52 ± 0.06	2.68 ± 0.04
High magnesium	4.12 ± 0.55	4.60 ± 0.13	2.18 ± 0.17	2.53 ± 0.06
High magnesium + high fluoride	3.13 ± 0.31 **	4.83 ± 0.28	1.67 ± 0.12 *	2.27 ± 0.14 *
		8 days		
Control	4.15 ± 0.41	4.88 ± 0.15	2.56 ± 0.05	2.96 ± 0.21
High fluoride	4.97 ± 0.20	4.34 ± 0.35	2.58 ± 0.06	2.52 ± 0.19
High magnesium	3.80 ± 0.55	4.96 ± 0.18	2.48 ± 0.35	3.14 ± 0.11
High magnesium + high fluoride	2.28 ± 0.31 **	5.50 ± 0.33	1.70 ± 0.36 *	2.86 ± 0.18
		10 days		
Control	5.14 ± 0.62	5.42 ± 0.07	2.54 ± 0.25	3.06 ± 0.07
High fluoride	5.33 ± 0.33	4.88 ± 0.39	2.47 ± 0.20	2.85 ± 0.24
High magnesium	3.35 ± 0.22 *	5.17 ± 0.17	2.51 ± 0.42	2.92 ± 0.03
High magnesium + high fluoride	2.57 ± 0.50 *	6.16 ± 0.38	2.09 ± 0.05	3.14 ± 0.33

[1] Average of 5 ± SE.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

^{45}Ca at a greater rate than the more calcified bones of the control group. The data on femur calcium levels, and the ^{45}Ca incorporation data appear to follow different trends in the older chicks, because the femur calcium levels are influenced by factors affecting calcium deposition in bone during the entire growth period, but the ^{45}Ca incorporation data are influenced by factors affecting the deposition only during the 4-hour incorporation period.

That increased mineral resorption is probably not responsible for the decreased femur calcium and phosphorus levels in the combination group is supported by the data in table 2. If an increased rate of mineral resorption had occurred in the high magnesium plus high fluoride group, one might expect to find a decrease in femur ^{45}Ca with an increase in time after injection. On the contrary, a slightly greater amount of isotope was present in the femurs of all four groups at 6 days compared with 4 hours after injection. Thus, it appears that increased mineral resorption is not an important factor involved in the magnesium plus fluoride inhibition.

The possibility that impaired intestinal absorption of calcium is responsible for the decreased mineralization in the combination group can be eliminated by considering the data in table 3. Although ^{45}Ca in-

TABLE 7

Plasma calcium and ^{45}Ca levels 4 hours after intraperitoneal dose as related to dietary treatment and chick age

Treatment	Days on treatment					
	2	3	4	6	8	10
Control Ca [1]	107 ± 1 [2]	104 ± 4	105 ± 2	116 ± 2	125 ± 9	136 ± 8
^{45}Ca [3]	1.21 ± 0.07	1.12 ± 0.09	1.06 ± 0.07	0.80 ± 0.03	0.70 ± 0.04	0.61 ± 0.08
High fluoride Ca	107 ± 3	110 ± 3	103 ± 4	112 ± 3	121 ± 3	122 ± 5
^{45}Ca	1.75 ± 0.05 **	1.32 ± 0.07	1.37 ± 0.10 *	1.06 ± 0.09 *	0.92 ± 0.08 *	0.73 ± 0.07
High magnesium Ca	102 ± 3	108 ± 5	96 ± 4	112 ± 3	121 ± 3	111 ± 1 *
^{45}Ca	1.37 ± 0.26	1.54 ± 0.10 *	1.22 ± 0.12	1.12 ± 0.04 **	0.74 ± 0.07	0.80 ± 0.07
High magnesium + high fluoride Ca	101 ± 2 *	107 ± 4	102 ± 4	116 ± 6	112 ± 2	111 ± 2 *
^{45}Ca	3.30 ± 0.13 **	4.69 ± 0.22 **	4.55 ± 0.25 **	2.77 ± 0.15 **	1.55 ± 0.01 **	1.53 ± 0.01 **

[1] Expressed as: μg/ml plasma.
[2] Average of 5 ± SE.
[3] Expressed as $\left(\frac{\%\ \text{dose}}{10\ \text{ml plasma}} \times \frac{\text{body wt}}{100}\right)$.
* Significantly different from corresponding control group, $P < 0.05$.
** Significantly different from corresponding control group, $P < 0.01$.

Fig. 1 Plasma ^{45}Ca specific activity of various times following intraperitoneal injection in 2-day-old chicks. Average of 5 ± SE. Control, ●——●; high fluoride, ●- - - -●; high magnesium, △——△; high magnesium + high fluoride, △- - - -△.

corporation into femurs of birds in the high magnesium plus high fluoride group is inhibited, absorption of the isotope from the gut was not reduced. Similar to what was observed following intraperitoneal injection, reduction in femur incorporation of ^{45}Ca in the high magnesium plus high fluoride group was accompanied by elevated plasma ^{45}Ca levels. From these data, it may be concluded that the primary factor involved in the magnesium × fluoride interaction is at the level of mineral uptake by bone.

The fact that plasma ^{45}Ca levels in the high magnesium plus high fluoride group remained elevated for 2 days following injection is probably a reflection of decreased bone turnover in this group (fig. 1). These results may be related to the observations

TABLE 8

Effect of dietary treatment on the incorporation of ^{45}Ca and ^{32}P into bone fragments in vitro [1]

Treatment	^{45}Ca [2]	^{32}P [2]
	% dose/g bone	*% dose/g bone*
Control	290 ± 8 [3]	171 ± 5 [3]
High fluoride	284 ± 8	157 ± 6
High magnesium	269 ± 8	157 ± 4
High magnesium + high fluoride	230 ± 6 **	128 ± 4 **

[1] Bone fragments from each of the four groups were incubated with pooled plasma from the respective groups for 2 hours at 41°. Chicks which had been fed the diets for 2 days were used.

[2] The data represent the average from three experiments; five chicks per treatment were used in each experiment.

[3] ± SE.

** Significantly different from corresponding control group, $P < 0.01$.

made by other workers (10, 11) that fluoride in tissue culture inhibits bone resorption. Also, by analogy one may speculate that since magnesium deficiency results in symptoms characteristic of hyperparathyroidism (12), i.e., hypercalcemia, hypophosphatemia and hyperphosphaturia, the combination treatment may result in an inhibition of parathyroid hormone activity. This might explain the failure of the elevated plasma ^{45}Ca levels in the high magnesium plus high fluoride group to return to the levels observed in the control group.

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Growth of Baby Pigs Fed Infant Soybean Formulas [1]

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ABSTRACT Newborn pigs were used to compare the nutritional quality of a recently developed soybean protein isolate (supplemented with methionine) with that of milk protein in 31-day studies. The pigs were fed liquid formulas which contained lactose as the sole carbohydrate and either 15% soybean protein calories or 10, 15, and 19% milk protein calories. Data from the pigs fed the milk formulas showed that weight gain and caloric efficiency, and levels of hemoglobin, plasma protein, liver protein, and carcass protein were directly related to protein content of the formulas; liver glycogen was inversely related to the level of protein in the diet. Weight gain, caloric efficiency and other data indicated that the protein quality of the soybean isolate plus methionine was approximately 85% that of milk protein.

Young baby pigs were used to compare the overall nutritional quality of an infant formula made from the soybean protein isolate with hitherto available infant soybean formulas made from soybean flour. During a 2-week pretest period, newborn pigs were fed milk formulas, either high or low in protein; they were then fed the soybean formulas for 4 weeks. In pigs fed a low protein pretest formula, marked differences in overall nutritional quality of the soybean formulas were found. The soybean isolate formula was far superior to the others. The pigs fed the high protein milk formula during the pretest period apparently had good protein reserves and little difference in growth performance was found on the soybean formulas. The latter studies show the importance of protein status of the test animal in short-term nutritional studies.

Newborn pigs fed infant liquid milk-based formulas grow moderately well and provide useful data for evaluating the overall nutritional value of these formulas (1). The present paper describes the use of the baby pig for evaluating the nutritional quality of infant soybean formulas. These studies were especially designed to assess the protein quality of a new soybean protein isolate supplemented with methionine, and to compare the overall nutritional quality of an infant formula made from this isolate with those made from soybean flour.

Work of several investigators suggests that newborn [2] or very young pigs do not grow well on infant soybean formulas because they lack the intestinal enzymes necessary to utilize either the crude soybean protein (2, 3) or the carbohydrates (4, 5) found in these formulas at an early age. Glucose may be used with isolated soybean proteins in feeding newborn pigs (6, 7), but since the milk of the sow contains lactose, the latter was used as the sole carbohydrate in the present studies comparing the protein quality of the soybean isolate with that of milk protein from the cow. Under these conditions, the soybean isolate was well utilized by the newborn pig, and the protein quality of the soybean isolate plus methionine was found to be approximately 85% that of milk protein.

In part 2 of the studies, to compare the nutritional value of the infant formula made from this soybean isolate with those of other marketed infant soybean formulas, some of which contain sucrose and complex soybean carbohydrates, newborn pigs were first fed lactose-containing milk formulas for a 2-week pretest period to allow time for sufficient development of other intestinal enzymes. (Previous studies have shown that newborn pigs did not survive when fed from birth a soybean isolate formula containing only sucrose as the carbohydrate.) [3] When the milk formula fed during the first 2 weeks was low in protein, subsequent feeding of the four soybean formulas showed marked differences

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[1] Presented in part at the Western Hemisphere Nutrition Congress II, San Juan, Puerto Rico, 1968.

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TABLE 1
Proximate analysis of formulas [1]

	Soybean isolate studies (exps. 1 and 2)				Infant soybean formula studies (exps. 3 and 4)					
					Pretest formulas		Test formulas			
	10M	15M	19M	15S	A [2]	B [3]	C	D	E	F
Type of protein	milk	milk	milk	soybean isolate	milk	milk	soybean isolate	soybean flour	soybean flour	soybean solids
Solids, g/liter	267	276	275	256	250	201	273	275	262	258
Protein, g/liter	35	52	68	50	33	62	53	62	59	38
Fat, g/liter	71	69	71	61	75	75	70	52	71	76
Carbohydrate, g/liter [4]	153	147	127	136	135	56	140	146	113	135
Ash, g/liter	8.6	8.1	10.0	9.2	6.8	8.8	10.0	11.2	12.3	6.7
Fiber, g/liter								4.6	6.7	2.6
Kcal/liter	1390	1420	1420	1295	1350	1150	1400	1300	1325	1375
Distribution of calories, %										
Protein	10	15	19	15	10	22	15	19	18	11
Fat	46	44	45	43	50	59	45	36	48	50
Carbohydrate	44	41	36	42	40	19	40	45	34	39

[1] Average of duplicate analyses. [2] Enfamil with Iron, Mead Johnson and Company, Evansville, Ind. [3] Similar to formula E (8). [4] Estimated by difference.

in nutritional quality of the formulas; when the pretest formula was high in protein, protein reserves were apparently increased and little difference in nutritional value of the soybean formulas was found.

EXPERIMENTAL

The animals used in these experiments were hysterectomy-obtained SPF (Specific Pathogen-Free) Hampshire-Yorkshire crossbred pigs.[4] The special facilities and techniques for handling the baby pigs and methods of analyses were reported previously (1, 8). Serum albumin was determined by electrophoresis.[5]

Nutritional value of soybean isolate. Two 31-day studies (exps. 1 and 2) were conducted with a total of 27 pigs from four litters. Newborn pigs were distributed into groups on the basis of weight, sex, and litter. These groups were fed four specially prepared formulas with lactose as the sole carbohydrate. Three contained protein from cow's milk at 10, 15, and 19% protein calories, respectively (formulas 10M, 15M, and 19M), and one contained the soybean isolate [6] (supplemented with 8 mg DL-methionine/g protein) providing 15% of the calories as protein (formula 15S). Their proximate analysis is shown in table 1. The milk protein formulas served as standards; formula 19M was used in only one study. Changes in protein content were made at the expense of lactose. The type of fat and levels of minerals and vitamins in these formulas were approximately the same as those in the infant formula made from the soybean isolate (formula C, table 1).

Comparison of infant soybean formulas. Two experiments were carried out (exps. 3 and 4). In experiment 3, newborn pigs were fed a pretest formula of concentrated commercial milk-base infant formula which supplied only 10% protein calories and 1350 kcal/liter (formula A, table 1). At 17 days of age, 15 pigs from two litters were distributed into four groups on the basis of weight, sex, and litter, and fed the commercially available

[4] Purchased from Pure-For-Sure, Flora, Ind.
[5] Paper electrophoresis system, model R, Beckman Instruments, Inc., Fullerton, Calif.
[6] Edi-Pro-N, Ralston Purina Company, St. Louis, Mo.

infant soybean formulas, C, D, E, and F [7] for 4 weeks (table 1). The caloric density of these formulas varied from 1300 to 1400 kcal/liter. The mineral and vitamin content of the four formulas differed somewhat but on the basis of label claim, all were considered adequate for the pig.

In experiment 4, newborn pigs were fed a pretest formula of simulated sow's milk containing 22% milk protein calories and 1150 kcal/liter (formula B, table 1). This formula gave good growth of baby pigs in previous studies (8). At 14 days of age, 20 pigs from three litters were distributed into four groups on the basis of weight, sex, and litter and fed the infant soybean formulas for 4 weeks as in the previous experiment.

RESULTS

Nutritional value of soybean isolate. The data from the two studies on formulas 10M, 15M, and 15S were combined on a weighted means basis and used to estimate the protein value of the soybean isolate. The data on formula 19M were from only one study, but fit well with the other data; the protein content of this formula approaches the level in sow's milk, i.e., 22% protein calories (8).

Weight gains of the groups fed formulas 10M, 15M, and 19M were markedly different within 1 week and were significantly different by the end of the experiment (table 2). Weight gain of the group fed the soybean isolate formula 15S was significantly lower than that found in the pigs fed the same level of milk protein, formula 15M. When the weight gain data of the group fed the soybean isolate are compared with those of pigs fed the milk-based formulas, plotted on the basis of protein content (fig. 1a), the 15% soybean protein calories are equivalent to 12.4% milk protein calories. Thus, the isolate has 83% of

[7] The formulas studied were Prosobee and Sobee (Mead Johnson and Company, Evansville, Ind.), Mull-Soy (The Borden Company, New York), and Soyalac (Loma Linda Foods, Riverside, Calif.).

TABLE 2

Weight gain, food intake, and blood data of pigs fed milk protein and soybean protein isolate formulas for 31 days (exps. 1 and 2)

	Formulas			
	10M	15M	19M	15S
No. of animals	6	7	6	8
Birth wt, kg	1.1	1.0	1.1	1.1
Cumulative wt gain, kg				
Day 7	0.3	0.6	0.7	0.5
Day 14	0.6	1.4	2.6	0.8
Day 21	1.4	3.4	6.0	2.2
Day 28	2.7	6.5	9.9	4.5
Day 31	3.5 ± 1.4 [1]	8.0 ± 1.8	11.5 ± 0.9	5.6 ± 1.4 [2]
31-day food consumption				
Formula, liters	22.2	36.8	50.4	30.5
Formula, solids, kg	5.9	10.1	13.9	7.8
Grams gained per 1000 kcal	110 ± 14	155 ± 15	161 ± 9	143 ± 23
Grams gained per gram protein	4.4 ± 0.5	4.2 ± 0.5	3.4 ± 0.2	3.7 ± 0.6
Blood data				
Plasma protein, g/100 ml				
15 day	3.4	3.7	4.5	4.0
31 day	3.7 ± 0.9	5.0 ± 0.7	5.2 ± 0	4.3 ± 1.0
Hematocrit, %				
Birth	24.7	19.8	20.7	23.6
31 day	33.7 ± 5.0	38.6 ± 6.5	44.4 ± 4	34.5 ± 4.3
Hemoglobin, g/100 ml				
Birth	7.6	6.6	6.3	7.6
31 day	9.4 ± 1.1	10.6 ± 0.7	13.0 ± 1	9.2 ± 1.2 [2]

[1] SD.
[2] $P < 0.02$ compared with formula 15M.

the growth-promoting activity of milk protein.

Total food intake increased as the level of protein in the formula increased, indicating the need of the faster growing pigs to consume more food (table 2). Caloric efficiencies (gram gain per 1000 kcal) also improved with increased levels of protein in the formula (table 2). The group fed the soybean isolate formula 15S gained 143 g/1000 kcal, equivalent to that found with 13.4% milk protein calories, or 89% of the value with milk protein (fig. 1b).

Two other measurements which show similar nutritional values for the soybean isolate are plasma and liver protein levels. Final (31 day) plasma protein levels increased as the level of protein in formulas 10M, 15M, and 19M increased (table 2); the average for the group fed the 15% soybean isolate formula was 4.3 g/100 ml, equivalent to 12.3% milk protein calories, or 82% of the value for milk protein (fig. 1c). Similarly, total liver protein levels reflected the level of protein in the milk formulas; observations with 15% soybean isolate were equivalent to those with 13.4% milk protein calories, or 89% of the effectiveness of milk protein (table 3, fig. 1d).

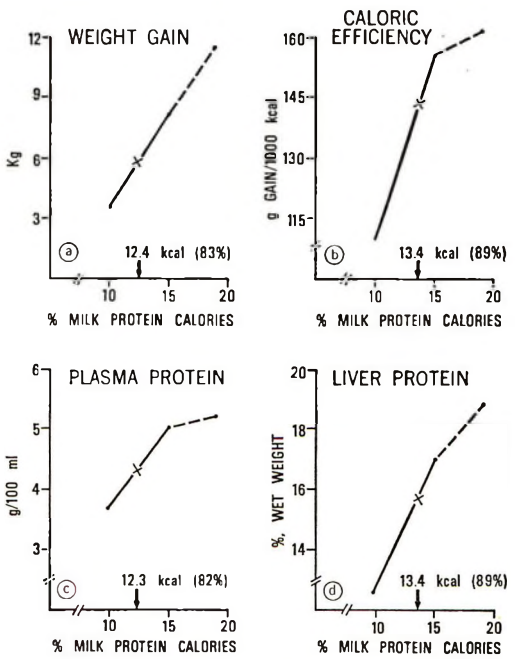

Fig. 1 Comparison of nutritive quality of soybean protein isolate with that of cow's milk protein. In each graph the value found with 15% soybean isolate protein calories (X) is used to estimate the equivalent milk protein activity (↓).

Protein utilization (grams gained per gram protein) decreased with the higher levels of milk protein (table 2). The difference in protein utilization between the soybean isolate and milk protein fed at the same level of calories was not significant.

Final hematocrit and hemoglobin values in the groups fed the milk protein formulas were also related to the level of protein in the formulas; the values for the group fed the soybean isolate were nearer to those found with the milk formula containing 10% protein calories (10M) than to those with the formula containing 15% protein calories (15M, table 2).

Relative organ weights (grams per kilogram body weight) were affected by the level of protein in the formulas; heavier liver, heart, and adrenal weights were found in the groups fed the lower protein-calorie formulas (table 3). This was apparently due to the lighter body weights of these groups, and indicated the precedence that organ development takes over growth. Organ weights in the pigs fed the soybean isolate formula (15S) were quite similar to those found in the pigs fed the same level of milk protein (15M).

In the livers, the protein levels were directly related to the protein level in the formulas as discussed above, and glycogen levels were inversely related to dietary protein level (table 3). Dietary protein level had little effect on liver fat and ash levels.

Carcass protein levels increased as the level of milk protein increased, with concomitant decreases in the levels of fat and ash (table 3). Values found for the pigs fed the soybean isolate formula 15S fell between those fed the 10M and 15M formulas.

Comparison of infant soybean formulas. In the first of these experiments, newborn pigs were fed the low protein milk formula A during the 17-day pretest period (table 1). They gained only 1.8 kg (table 4) and apparently had low protein reserves based on observations with 10% milk protein (formula 10M) in table 2. After 4 weeks (17 to 45 days of age) of feeding the four infant soybean formulas, weight gain and

TABLE 3

Organ weight, liver and carcass composition of pigs fed milk protein and soybean protein isolate formulas for 31 days (exps. 1 and 2)

	Formulas			
	10M	15M	19M	15S
No. of animals	6	7	6	8
Final body wt, kg	5.6	9.9	12.8	8.2
Organ weights				
Liver wt, g	220	332	352	288
Liver, g/kg body wt	40.3± 4.6 [1]	34.3± 5.4	27.4± 3.3	35.2± 1.9
Heart wt, g	35.5	57.8	72.0	47.4
Heart, g/kg body wt	6.6± 1.0	6.0± 0.7	5.6± 0.2	5.9± 0.7
Kidneys wt, g	38.7	74.9	98.2	59.8
Kidneys, g/kg body wt	7.2± 1.1	7.7± 0.9	7.6± 0.7	7.4± 1.2
Adrenals wt, mg	685	945	1034	792
Adrenals, mg/kg body wt	139 ±55	103 ±40	81 ±13	105 ±47
Liver composition				
Solids, %	25.1± 1.3	25.0± 0.7	25.1± 0.6	24.0± 1.0
Fat, %	2.8± 0.3	3.3± 0.2	3.7± 0.2	3.2± 0.3
Protein, %	12.6± 2.6	17.0± 1.6	18.8± 0.2	15.7± 2.1
Ash, %	1.3± 0.4	1.5± 0.3	1.4± 0.1	1.6± 0.5
Glycogen, %	7.7± 2.5	3.6± 1.8	1.3± 0.7	3.8± 2.3
Carcass composition [2]				
Solids, %	41.6± 6.1	41.1± 1.3	41.3± 2.4	39.2± 2.3
Fat, %	25.7± 6.6	24.5± 2.3	23.5± 2.8	23.0± 2.3
Protein, %	12.1± 0.5	14.1± 0.5	14.9± 0.4	13.1± 0.5 [3]
Ash, %	2.7± 0.4	2.0± 0.2	2.1± 0.3	2.3± 0.1 [3]

[1] SD.
[2] Carcass minus gastrointestinal tract and liver.
[3] $P < 0.01$ compared with formula 15M.

caloric efficiency (gram gain per 1000 kcal) of the pigs fed formula C containing the soybean isolate were significantly higher than those found with the other soybean formulas (table 4).

Protein utilization ranged from 1.9 to 3.5 g gained/g protein (table 4) with the highest values found on formula C. Since protein utilization increased on low protein formulas (table 2), the 3.5 g gained/g protein with the 15% protein-calorie soybean isolate formula C must be considered superior to the 3.4 g gained on the 11% protein-calorie formula F.

Final serum protein levels were also highest in the group fed formula C; the levels in the group fed formula F were significantly lower than those in all other groups, probably reflecting the lower protein content of this formula (table 4). Serum albumin levels followed the same trend, but only the difference between the groups fed formulas C and F was significant. Hematocrit and hemoglobin levels did not differ markedly, but values for the group fed formula E were lowest.

Differences in relative organ weights (table 5) were related to both level and quality of protein in the formulas. Except for relative liver weights of the group fed formula E, organ weights of the pigs fed formulas D, E, and F were higher than those of pigs fed formula C (table 5). Liver composition varied little in the four groups.

Carcass analysis showed little difference in levels of protein, fat, and ash in the four groups (table 5). Total protein in the carcass of the group fed the soybean isolate formula C, however, was considerably higher than that of pigs fed the other formulas.

In experiment 4, the newborn pigs were fed simulated sow's milk (22% protein calories) during the 14-day pretest period. They gained weight rapidly and presumably had good protein reserves (table 6). After the pigs received the four infant soybean formulas for 4 weeks (14 to 42 days of age), there were no significant differences in weight gains as were found in experiment 3 (table 4). Caloric efficiency

TABLE 4

Weight gain, food intake, and blood data of 17-day-old pigs fed infant soybean formula products for 4 weeks (exp. 3)

Pretest period (17 days)	Formula A
No. of animals	15
Birth wt, kg	1.2
17 day wt gain, kg	1.8 ± 0.3 [1]
Formula consumed, liters	12.2

Test period (28 days)	Infant soybean formula products			
	C	D	E	F
No. of animals	4	4	3	4
17 day wt, kg	3.0	3.0	2.9	3.0
17 to 45 day wt gain, kg	16.6 ± 1.9	10.9 ± 1.7 [2]	9.7 ± 1.6 [2]	7.7 ± 2.1 [2]
17 to 45 day food consumption				
Formula, liters	92.5	85.7	83.6	61.7
Formula solids, kg	24.8	23.5	21.9	15.7
Grams gained per 1000 kcal	130 ± 12	98 ± 9 [2]	87 ± 12 [2]	90 ± 11 [2]
Grams gained per gram protein	3.5 ± 0.3	2.0 ± 0.2 [2]	1.9 ± 0.3 [2]	3.4 ± 0.4 [3]
Blood data				
Serum protein, g/100 ml				
17 day	3.2	3.1	2.7	2.6
45 day	5.0 ± 0.5	4.6 ± 0.5	4.4 ± 0.4	3.9 ± 0.1 [4]
Serum albumin, g/100 ml				
17 day	1.2	1.2	1.0	0.9
45 day	2.8 ± 0.3	2.7 ± 0.5	2.6 ± 0.3	2.2 ± 0.2 [5]
Hematocrit, %				
17 day	28.5	30.1	25.9	28.1
45 day	36.6 ± 5.2	36.7 ± 2.2	29.0 ± 3.1 [6]	35.0 ± 0.4
Hemoglobin, g/100 ml				
17 day	8.7	9.2	8.7	8.9
45 day	11.1 ± 1.6	11.3 ± 0.7	9.0 ± 0.8 [6]	11.3 ± 0.3

[1] SD.
[2] $P < 0.01$ compared with formula C.
[3] $P < 0.01$ compared wtih formulas D and E.
[4] $P < 0.05$ compared with other formulas.
[5] $P < 0.02$ compared with formula C.
[6] $P < 0.02$ compared with formulas D and F.

(gram gain per 1000 kcal) of the soybean isolate (formula C), however, was considerably better than that of formulas D, E, and F. Protein utilization (gram gain per gram protein) was also better than found with formulas D and E and presumably with formula F based on dietary protein levels (table 6).

There were no significant differences in blood values, relative organ weights, or liver composition; these data are not shown.

Carcass composition varied considerably between the groups (table 6). In the pigs fed formulas E and F, levels of carcass solids and fats were significantly higher and carcass protein somewhat lower than were found in the pigs fed formulas C and D. Total carcass protein laid down in the group fed the soybean isolate (formula C) was considerably greater than in the other groups.

DISCUSSION

Reasonably good growth was obtained in colostrum-deprived newborn pigs fed the soybean protein isolate (plus methionine) formula with lactose as the carbohydrate. Somewhat similar results were obtained by other investigators feeding C-1 assay soybean protein in a liquid formula containing glucose to newborn (7) and as

TABLE 5

Organ weight, liver and carcass composition of 17-day-old pigs fed infant soybean formula products for 4 weeks (exp. 3)

	Infant soybean formula products			
	C	D	E	F
No. of animals	4	4	3	4
Final body wt, kg	19.8	14.0	12.8	10.6
Organ weights				
Liver, g	521	391	330	296
Liver, g/kg body wt	26.2 ± 0.8 [1]	28.0 ± 1.1 [2]	25.9 ± 1.2	28.4 ± 3.4
Heart, g	97.9	84.5	79.1	62.7
Heart, g/kg body wt	4.9 ± 0.3	6.1 ± 0.6 [2]	6.2 ± 0.3 [2]	6.1 ± 1.0
Kidneys, g	112.3	121.6	105.2	62.5
Kidneys, g/kg body wt	5.7 ± 0.8	8.7 ± 0.9 [2]	8.3 ± 0.5 [2]	6.0 ± 0.9
Adrenals, mg	1270	1217	973	1022
Adrenals, mg/kg body wt	64.5 ±16.4	88.5 ±21.0	77.3 ±13.2	99.7 ±21.3 [2]
Liver composition				
Solids, %	25.3 ± 0.6	25.8 ± 0.8	25.7 ± 0.7	24.8 ± 0.6
Fat, %	4.1 ± 0.0 [3]	4.6 ± 0.3	4.3 ± 0.3	4.5 ± 0.1
Protein, %	18.6 ± 0.8	19.8 ± 0.7	19.3 ± 0.9	19.6 ± 0.4
Ash, %	1.4 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.4 ± 0.1
Glycogen, %	1.3 ± 1.1	0.6 ± 0.2	1.1 ± 0.8	0.1 ± 0.0
Carcass composition [4]				
Solids, %	50.4 ± 3.6	46.3 ± 2.7	45.2 ± 2.6	51.8 ± 5.1
Fat, %	36.2 ± 4.8	30.8 ± 3.2	29.3 ± 3.6	37.7 ± 6.8
Protein, %	11.9 ± 1.4	13.4 ± 0.7	13.0 ± 0.5	11.4 ± 1.8
Ash, %	1.8 ± 0.4	1.5 ± 0.2	1.8 ± 0.4	1.6 ± 0.3
Total protein, g	2051 ±379	1547 ±252	1373 ±194	1028 ±366

[1] SD.
[2] $P < 0.05$ compared with formula C.
[3] $P < 0.02$ compared with formulas D and F.
[4] Carcass minus gastrointestinal tract and liver.

a gruel to pigs a few days old (9). These studies show that with proper attention to carbohydrate and other nutrients in the formula, it should be possible to gain useful data about the quality of protein from sources other than milk in the newborn pig, the age most desirable for studies relating to the human infant.

The formulas used in these studies had a slightly higher caloric density than sow's milk, but the levels of protein calories were below those required for maximum growth in the baby pig. Thus, the milk protein formulas provided a useful curve against which the nutritive value of the soybean isolate could be compared. All of the parameters which were indicative of nutritive quality showed that the soybean isolate formula containing 15% protein calories was equivalent to approximately 12.3 to 13.5% milk protein-calorie formulas, and indicate that the soybean isolate plus methionine has about 85% of the nutritive value of milk protein. In the rat it is 97% of the protein efficiency ratio (PER) value for casein (10).

The differences found with the formulas containing the graded levels of milk protein suggest several useful parameters such as weight gains, plasma proteins and liver and carcass composition by which the baby pig can be used as a tool in evaluating infant liquid formulas. The inverse relationship between liver protein and glycogen levels appears to be related to the quantity or quality, or both, of dietary protein and warrants further study. The reason for this inverse relationship is unexplained but may simply be a compensatory mechanism. Other investigators (11, 12) have shown that the pig stores liver glycogen when fed low protein diets. Wid-

TABLE 6

Weight gain, food intake and blood data of 14-day-old pigs fed infant soybean formula products for 4 weeks (exp. 4)

Pretest period (14 days)	Formula B
No. of animals	20
Birth wt, kg	1.3
14 day wt gain, kg	2.4 ± 0.2 [1]
Formula consumed, liters	11.0

Test period (28 days)	Infant soybean formula products			
	C	D	E	F
No. of animals	5	5	5	5
14 day wt, kg	3.7	3.7	3.7	3.7
14 to 42 day wt gain, kg	12.7 ± 1.0	11.0 ± 3.3	12.2 ± 3.1	11.0 ± 1.5
14 to 42 day food consumption				
Formula, liters	72.8	85.3	84.9	85.9
Formula, solids, kg	20.3	23.6	23.7	22.4
Grams gained per 1000 kcal	123.0 ± 13.0	100.0 ± 7.0 [2]	109.0 ± 9.0	93.0 ± 11.0 [2]
Grams gained per gram protein	3.2 ± 0.3	2.1 ± 0.2 [3]	2.5 ± 0.2 [3]	3.3 ± 0.1
Carcass composition [4]				
Solids, %	44.7 ± 2.8	44.5 ± 4.3	47.0 ± 2.4 [2]	55.4 ± 1.6 [5]
Fat, %	28.1 ± 3.7	28.3 ± 5.1	31.2 ± 2.7	42.3 ± 1.8 [5]
Protein, %	14.0 ± 0.5	14.3 ± 1.0	13.2 ± 0.8	11.8 ± 1.0 [5]
Ash, %	2.3 ± 0.3	1.9 ± 0.1	2.0 ± 0.4	1.7 ± 0.3
Total protein, g	1955 ± 198	1697 ± 309	1722 ± 366	1434 ± 155

[1] SD.
[2] $P < 0.01$ compared with formula C.
[3] $P < 0.01$ compared with formulas C and F.
[4] Carcass minus gastrointestinal tract and liver.
[5] $P < 0.01$ compared with formulas C and D.

dowson (13) found that newborn pigs have higher levels of liver glycogen than usually found in most laboratory animals.

The differences between the observations in the two studies on the infant soybean formulas demonstrate the effect that protein reserve status can have on subsequent evaluation of formulas. The pig appears to have the capacity to protect itself for some period of time against poor protein nutrition (14). Thus, as found in pigs (15) and also in rats (16), animals with limited protein reserves probably give a better estimate of protein quality than do protein-sufficient animals. Observations in pigs prefed the low protein formula are in agreement with protein efficiency values for these soybean formulas in the rat (10).

The superior nutritive value of the infant formula made with the soybean isolate is due partially to the addition of methionine to the formula, but it may also be related in part to the removal of complex carbohydrate and other flatus-producing compounds found in soybeans (17, 18).

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Bile Salt Enhancement of Riboflavin and Flavin Mononucleotide Absorption in Man [1]

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ABSTRACT The gastrointestinal absorption of riboflavin and flavin mononucleotide (FMN) was determined under control conditions and after oral administration of 600 mg sodium deoxycholate. When the bile salt is given prior to a 30-mg dose of riboflavin there is a 50 to 80% increase in total urinary recovery of apparent riboflavin. A similar, but less marked, enhancement is observed when the same dose of FMN is given with sodium deoxycholate. Urinary excretion data also suggest an unusually prolonged absorption of riboflavin in the presence of the bile salt. The possibility exists that the bile salt enhancement of riboflavin and FMN absorption may be due to changes in gastrointestinal motility or changes in the permeability of the gastrointestinal membranes to the transport of the vitamins, or both. Other possibilities are also explored.

Although the human nutritional requirement for riboflavin has been recognized for some time, speculation still exists concerning the gastrointestinal absorption of this vitamin. Morrison et al. (1) have suggested that riboflavin is absorbed high in the human intestinal tract based upon the observation that peak urinary excretion values were obtained within 2 hours after oral administration of riboflavin in solution or in sustained release form. Lane et al. (2) reported that the percentage of the administered dose of riboflavin recovered in the urine (which reflects the percentage of dose which is absorbed) following oral administration, decreased with increasing dose. The latter observation was confirmed by Stripp (3), who also showed that not more than 14 to 18 mg of riboflavin are excreted in the urine, independent of oral dose of riboflavin or flavin mononucleotide (FMN) when the dose exceeds 50 mg. More recently, Levy and Jusko studied the gastrointestinal absorption of riboflavin (4) and FMN (5) in man and suggested the existence of a specialized transport process for the vitamin in the upper region of the intestinal tract.

The absorption of several nutrients may be strongly influenced by bile salts, an important class of physiological surface active agents. Extensive research has indicated the importance of bile salts in lipid absorption (6–8). In addition, there have been several studies implicating bile salts in the absorption of water-insoluble vitamins. These reports demonstrate that the absorption of vitamins A (9–11), D (12–14) and K (15) is enhanced by the presence of various bile salts.

Bates et al. (16–18) have suggested, based on solubilization and dissolution studies of several water-insoluble drugs, that bile salts may influence drug absorption by enhancing dissolution rate. Cavallito and O'Dell (19) studied the influence of coadministration of sodium cholate and dehydrocholate on the pharmacologic response to a quaternary hypotensive agent in dogs. In each case an apparent increase in drug absorption was suggested by the enhanced pharmacologic response. Davenport (20) reported that sodium taurocholate and natural bile markedly disrupt the gastric mucosal barrier in dogs as evidenced by changes in ionic fluxes. Studies with isolated intestinal segments have shown the influence of bile salts on nutrient (21) as well as drug (22) transfer.

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A preliminary indication that bile salts may affect riboflavin absorption is the work of Onishi (23) who found that ursodeoxycholic acid significantly increases the absorption of esterified riboflavin as well as total riboflavin in excised dog intestine. The present study concerns the influence of an unconjugated bile salt, sodium deoxycholate, on the absorption of riboflavin and FMN in man.

EXPERIMENTAL

Five, apparently healthy, male volunteers, ranging in age from 24 to 29 years, served as test subjects. The urinary excretion of riboflavin was followed after oral administration of riboflavin or FMN under the following experimental conditions: 1) riboflavin,[4] 30 mg, with and without 600 mg sodium deoxycholate[5] (four subjects); 2) FMN,[6] equivalent to 30 mg riboflavin, with and without 600 mg sodium deoxycholate (five subjects); and 3) riboflavin, 5, 10, 20 and 30 mg with and without 600 mg sodium deoxycholate (one subject).

All subjects ingested the vitamin in the morning following an overnight fast. In studies involving administration of sodium deoxycholate, the bile salt was first dissolved in 100 ml of water. Ten grams of an artificial orange juice concentrate[7] were added to the solution to mask the taste of the bile salt and the resulting colloidal dispersion was ingested 0.5 hour prior to ingestion of the vitamin. In control studies, the equivalent amount of orange concentrate in 100 ml of water was administered 0.5 hour before the vitamin. Riboflavin or FMN was dissolved or partially dissolved[8] in 200 ml of water prior to ingestion.

Urine was collected immediately after ingestion of the vitamin and every half-hour for 2 to 3 hours thereafter, at hourly intervals for at least the next 4 hours, and then at convenient intervals up to 24 hours. Where possible, on the day before the experiment a 24-hour control urine collection was made. At least two such collections were carried out in each subject. Glacial acetic acid (about 3 ml/100 ml urine) was added to each urine sample and the urine was immediately refrigerated (protected from light) until assayed within 24 hours after termination of urine collection.

Subjects were instructed to drink a sufficient quantity of water after each voiding to maintain adequate urine volumes. In addition, the subjects were asked to avoid ingesting foods known to contain appreciable amounts of riboflavin as well as any vitamin preparations and other drugs. No food was ingested until at least 2 hours after administration of the vitamin. All paired experiments (i.e., control versus sodium deoxycholate) were performed in a random manner and at least 1 week elapsed between experiments in any one subject.

Total riboflavin in the urine was determined fluorometrically according to Levy and Jusko (4), using a Turner fluorometer.[9] Briefly, this method involves mixing 5 ml diluted urine with 1 ml of 1 N acetate buffer, pH 4.8. One milliliter of 4% potassium permanganate and, subsequently, 1 ml of 3% hydrogen peroxide are added. The fluorescence intensity of this solution is determined before and after reduction of riboflavin with sodium hydrosulfite. All data were corrected for blank values which averaged 0.5 mg/24 hours of apparent riboflavin. Apparent riboflavin excretion was unchanged during the 24-hour period following the oral administration of 600 mg sodium deoxycholate.

RESULTS

The influence of sodium deoxycholate on the 24-hour total urinary riboflavin recovery is summarized in table 1 and a representative plot is shown in figure 1. The control recovery values range from 13 to 20% of the 30-mg dose, in agree-

[4] Ruger Chemical Company, Long Island City, N. Y., lot no. 0069.

[5] Mann Research Laboratories, division of Becton, Dickinson and Company, New York, N. Y., lot no. T3932.

[6] Hoffmann-LaRoche, Inc., Nutley, N. J., lot no. 445016.

[7] Tang, General Foods Corporation, White Plains, N. Y.

[8] Flavin mononucleotide is soluble in the quantity of water administered. Riboflavin has a water solubility of about 12 mg/100 ml at room temperature.

[9] Turner fluorometer model 110, G. K. Turner Associates, Palo Alto, Calif.

TABLE 1

Total (24 hour) urinary excretion of riboflavin [1] after oral administration of 30 mg riboflavin

Subject	Control	SDC [2]	Ratio (SDC/control)
SF	20.2	30.2	1.50
WJ	13.5	22.5	1.67
MM	23.1	38.7	1.68
CN	15.0	26.7	1.78

[1] Expressed as percentage of dose.
[2] Sodium deoxycholate, 600 mg, administered 0.5 hour before the vitamin.

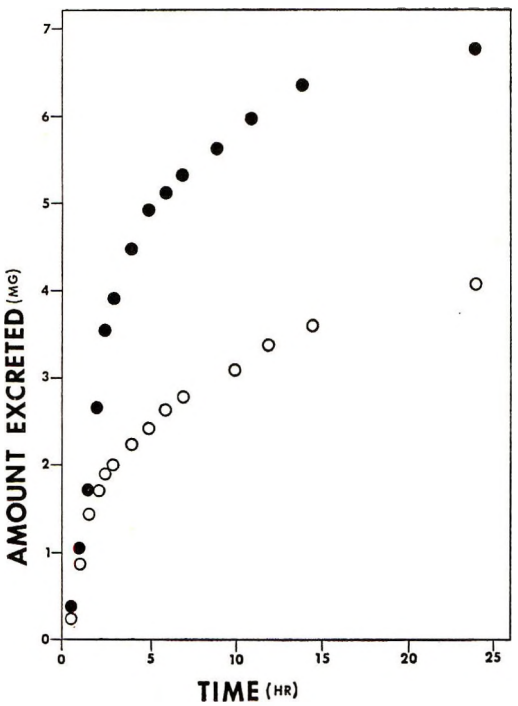

Fig. 1 Cumulative amount of riboflavin excreted after oral administration of 30 mg riboflavin (subject WJ). (○) Control; (●) sodium deoxycholate, 600 mg, administered 0.5 hour before the vitamin.

ment with the values reported by Levy and Jusko (4) for fasting subjects taking the same dose in solution. When sodium deoxycholate was given prior to riboflavin there was a 1.5- to 1.8-fold increase in total urinary recovery. A similar, but less marked enhancement was observed when the same dose of FMN was given with sodium deoxycholate, as shown in table 2 and figure 2.

The results were statistically analyzed by the method of paired comparisons using Student's *t* test (24). Table 3 shows the comparisons made and the resulting levels of significance. Total recovery of riboflavin after oral administration of riboflavin or FMN with bile salt is significantly greater than the respective control values. Although urinary recoveries of riboflavin after oral administration of either riboflavin or FMN did not differ significantly from each other, there was a high level of significance between urinary recoveries after riboflavin with sodium deoxycholate and FMN with sodium deoxycholate.

TABLE 2

Total (24 hour) urinary excretion of riboflavin [1] after oral administration of flavin mononucleotide (FMN) [2]

Subject	Control	SDC [3]	Ratio (SDC/control)
LA	27.9	31.6	1.13
SF	24.5	26.2	1.07
WJ	11.3	16.5	1.46
MM	14.0	24.3	1.74
CN	14.9	18.5	1.24

[1] Expressed as percentage of dose.
[2] Dose equivalent to 30 mg riboflavin.
[3] Sodium deoxycholate, 600 mg, administered 0.5 hour before the vitamin.

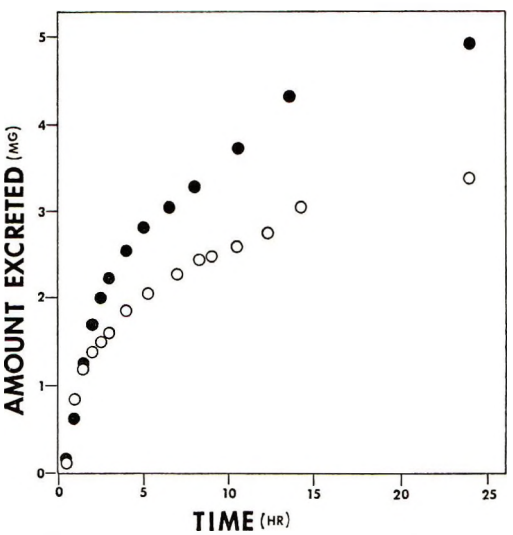

Fig. 2 Cumulative amount of riboflavin excreted after oral administration of FMN, equivalent to 30 mg riboflavin (subject WJ). (○) Control; (●) sodium deoxycholate, 600 mg, administered 0.5 hour before the vitamin.

Figure 3 shows a typical semilogarithmic plot of excretion rate of riboflavin as a function of time after oral administration of riboflavin with and without sodium deoxycholate. Administration of riboflavin with sodium deoxycholate results in a marked increase in the peak excretion rate compared with control values. Similar results are observed after administration of FMN. The data in figure 3 also indicate a considerable shift in the peak excretion rate after bile salt administration. Peak excretion rate of riboflavin occurs within 1 hour after oral administration of riboflavin, but only after about 2 hours when the vitamin is given with sodium deoxycholate. This shift is suggestive of a prolonged absorption of riboflavin in the presence of the bile salt. Shifts in peak excretion rates between control and sodium deoxycholate experiments were not as evident after FMN administration.

The decline in body levels of total flavins in the postabsorptive phase, as manifested by the decreasing excretion rates of riboflavin some time after administration, may be characterized by a rapid elimination and slow elimination phase, as

TABLE 3

Statistical analysis [1] of total urinary excretion of riboflavin after oral administration of riboflavin [2] or FMN [2] with and without sodium deoxycholate (SDC)

Comparison	1 versus 2		Level of significance
	mean % of dose excreted		
Riboflavin(1) versus riboflavin-SDC(2)	18.0	29.5	$P < 0.001$
FMN(1) versus FMN-SDC(2)	18.5	23.4	$P < 0.025$
Riboflavin(1) versus FMN(2)	18.0	16.2	$P > 0.1$
Riboflavin-SDC(1) versus FMN-SDC(2)	29.5	21.4	$P < 0.025$

[1] Paired comparison using Student's *t* test.
[2] Dose equivalent to 30 mg riboflavin.

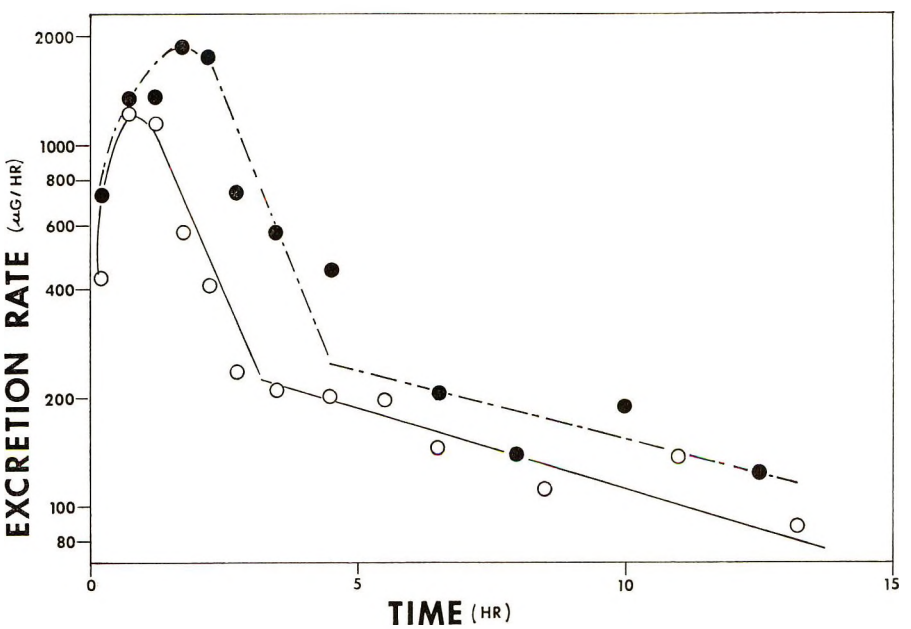

Fig. 3 Urinary excretion rate of riboflavin after oral administration of 30 mg riboflavin (subject WJ). (○) Control; (●) SDC.

noted in figure 3. The biexponential elimination of riboflavin has been noted and discussed previously by Levy and Jusko (4). In all experiments with FMN or riboflavin, with and without sodium deoxycholate, the rapid elimination phase yielded half-lives of 0.5 to 2 hours, while the slow elimination phase yielded half-lives of 4 to 7 hours. Paired analysis of the half-life data clearly indicated that sodium deoxycholate had no significant effect on the elimination kinetics of riboflavin after the administration of either riboflavin or FMN. Hence, one may conclude that differences in the excretion rate of riboflavin in the presence of sodium deoxycholate are the result of changes in the gastrointestinal absorption of the vitamins when administered with the bile salt.

Figure 4 shows a dose-response-type plot of the total amount of riboflavin ultimately excreted in the urine after various oral doses of riboflavin, with and without sodium deoxycholate. It is evident that there is a considerably increased urinary excretion of riboflavin at all dose levels when the bile salt is coadministered with the vitamin. It is also apparent that both curves tend to plateau as the dose increases. Levy and Jusko (4) have postulated that the latter phenomenon may be due to a specialized intestinal absorption process which is capacity limited.

DISCUSSION

In agreement with previous work (2–5), the present results demonstrate that the percentage of administered dose of riboflavin which is absorbed after oral administration, decreases with increasing dose. This finding is consistent with the hypothesis that the absorption of riboflavin involves a specialized transport which is capacity limited (4). There is no evidence that renal excretion of riboflavin is saturable in the concentration range found in the present study (4). Furthermore, in the dose range employed, there is essentially quantitative urinary recovery of absorbed riboflavin or FMN, as riboflavin (3, 4). It follows, therefore, that a Lineweaver-Burk-type plot of the reciprocal of the amount excreted versus the recipro-

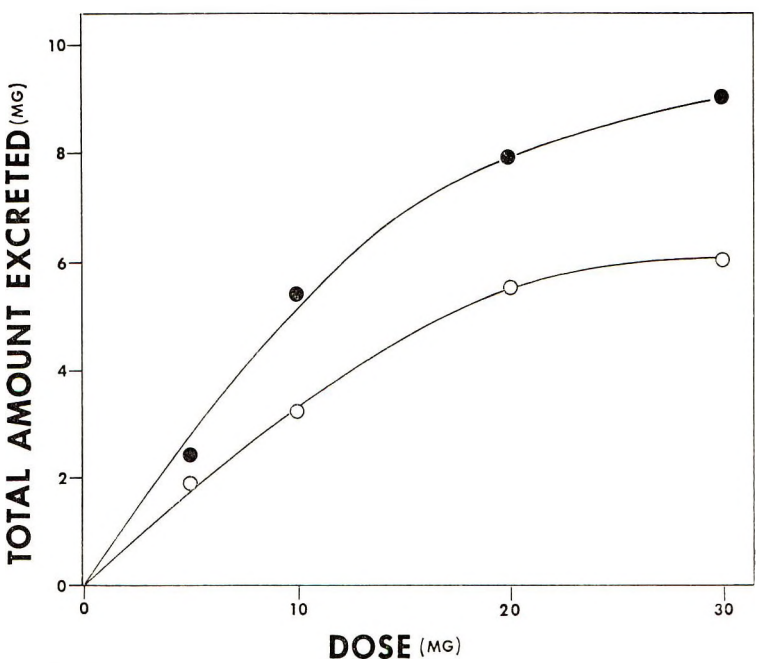

Fig. 4 Total amount of riboflavin excreted as a function of oral dose of riboflavin (subject SF). (○) Control; (●) SDC.

cal of the dose of riboflavin, should be linear (4). Evidence of this is shown in figure 5.

Dowd and Riggs (25), have noted that care must be taken in the interpretation of the usual Lineweaver-Burk-type plot since the double reciprocal plot offers the poorest estimate of the appropriate kinetic parameters. They propose that when dealing with values of "v" (which correspond to values of the amount recovered in the urine in the present study), where the error is unknown, it would be advantageous to plot "v" versus "v/S" (where S corresponds to the administered dose) to obtain the best values of K_m and V_{max}. An analogous plot using the riboflavin data is shown as an inset to figure 5. Unweighted least square regression analysis of both lines yields values of 12.1 and 11.3 mg as the maximum amount of riboflavin which can be absorbed from the gastrointestinal tract of the fasting subject, and values of 27 and 24.2 mg as the dose which yields an amount absorbed equal to one-half of the maximum, using the double reciprocal plot and the "total amount excreted versus total amount excreted per dose" plot, respectively. The two types of plots yield values which are in excellent agreement and tend to confirm the capacity-limited nature of intestinal riboflavin absorption. The urinary excretion data obtained after administration of various doses of riboflavin with sodium deoxycholate yielded poor fits in Lineweaver-Burk-type plots.

Previous studies in this laboratory suggest a number of possible mechanisms by which sodium deoxycholate may influence riboflavin and FMN absorption. If the absorption of a material is limited to the upper gastrointestinal tract, as is the case for both riboflavin and FMN (1, 2, 4), then the residence time of the material at the absorption sites is a critical determinant of the total amount of drug which can be absorbed. Feldman et al. (26)

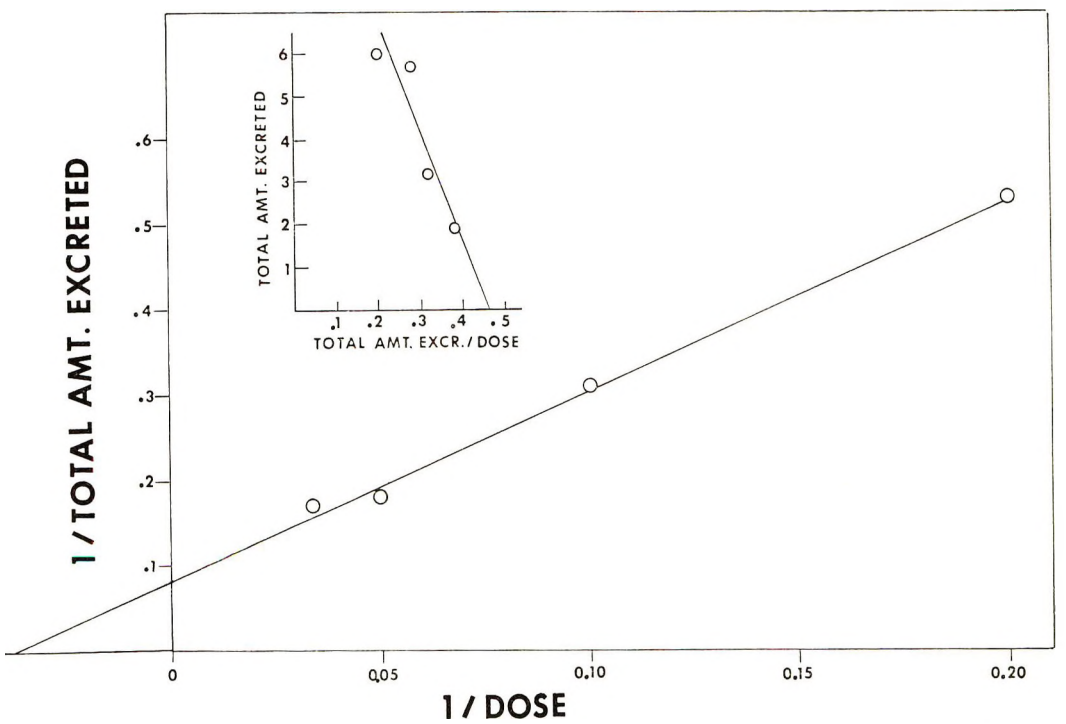

Fig. 5 Lineweaver-Burk-type plot of the riboflavin urinary excretion data as a function of oral dose, as discussed in the text (subject SF). Inset shows an alternate method of plotting the data.

and Feldman and Gibaldi (27) have recently shown that oral administration of sodium taurodeoxycholate and sodium deoxycholate markedly inhibits gastric emptying and proximal intestinal transit in the rat. Similar effects in man would result in prolonged and more complete absorption of the coadministered vitamins. This possibility is consistent with the data obtained after oral administration of riboflavin as shown in figure 3. The plot clearly suggests that the absorption of riboflavin is prolonged in the presence of sodium deoxycholate as evidenced by the shift in peak excretion rate. Levy and Jusko (4, 5) have shown marked increases in riboflavin and FMN absorption upon administration after a standard meal, in contrast to the absorption observed in fasting subjects. They attribute this enhancement to a decrease in gastric emptying due to the presence of food which causes the vitamins to be in contact with optimum absorption sites in the proximal region of the intestinal tract for a longer period of time.

Changes in the permeability of the gastrointestinal membranes due to sodium deoxycholate may also account in part for the marked increases in the absorption of riboflavin and FMN. The effects of bile salts on membrane permeability have been studied extensively (20, 22, 26, 28, 29). The possibility that administered bile salts increase the permeability of the gastrointestinal membranes to riboflavin is supported by current studies in this laboratory.[10] Figure 6 shows a plot of the rate of transfer of riboflavin across the isolated everted rat intestine determined according to the method of Feldman and Gibaldi (22), in the presence and absence of 10 mM sodium taurodeoxycholate. A sig-

[10] Mayersohn, M., and M. Gibaldi, unpublished data.

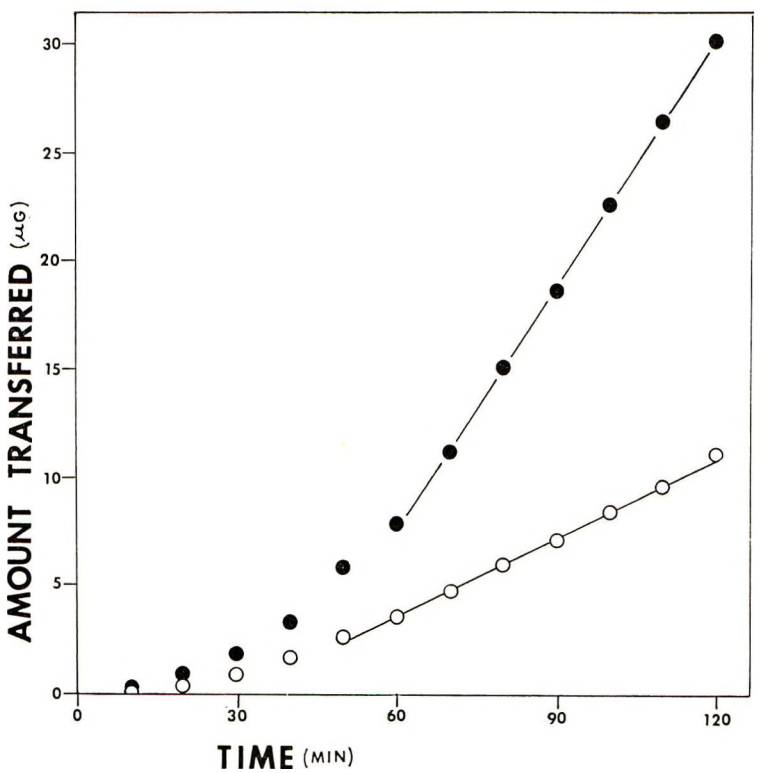

Fig. 6 Mucosal-to-serosal riboflavin transfer across the everted intestine of the rat. (○) Control; (●) sodium taurodeoxycholate, 10 mM. Mucosal solution concentration maintained constant at 20 μg/ml riboflavin.

nificant increase in the transfer rate of riboflavin in the presence of the bile salt is apparent.

In vitro studies [11] indicate that sodium deoxycholate can solubilize riboflavin in water. Approximately 156 moles of bile salt are required to solubilize 1 mole of riboflavin. Hence, the possibility must be considered that the detergent action of sodium deoxycholate simply enhances the solubility of riboflavin in the gastrointestinal tract as with certain fat-soluble vitamins. Though FMN is considerably more water soluble than riboflavin, it must first be dephosphorylated, and then the resultant riboflavin may require further solubilization before absorption.

Another possible effect of sodium deoxycholate could be on the intestinal microflora. Yang and McCormick (30) present evidence to suggest degradation of riboflavin by the intestinal microorganisms. Inhibition of these microorganisms, for example by deoxycholate, may result in a larger fraction of drug absorbed.

A particularly interesting observation in the present study, which cannot be explained at present, is the difference in effect of sodium deoxycholate on riboflavin and FMN absorption. As shown in figures 1 and 2, and table 3, the effect of sodium deoxycholate on riboflavin absorption was significantly greater than its effect on FMN absorption. Levy and Jusko (4, 5) present evidence which suggests that riboflavin and FMN are absorbed by the same specialized transfer process. There is extensive evidence showing that FMN is rapidly and completely dephosphorylated to free riboflavin in the small intestine, which explains the similarity in the absorption of riboflavin and FMN noted by Levy and Jusko (4, 5), and in the present study in the absence of bile salt. A possible explanation for the differences in absorption between riboflavin and FMN in the presence of sodium deoxycholate may be the influence of the bile salt on the dephosphorylating enzyme system. Unconjugated bile salts have been found to be very active inhibitors of a number of enzyme systems in the intestine (21), and may also depress the conversion of the phosphate ester to free riboflavin. A further possibility which cannot be ruled out is that significant amounts of FMN may be excreted in the urine after administration of deoxycholate. This would be the situation if a sufficient amount of bile salt reached the systemic circulation to inhibit dephosphorylation. Since the assay procedure is not specific for riboflavin, the results would suggest a lower amount of "apparent" riboflavin than the true amount of total flavins.

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Effectiveness of Various Sources of Nonessential Nitrogen in Promoting Growth of Chicks Fed Carbohydrate-containing and "Carbohydrate-free" Diets [1]

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ABSTRACT Studies were conducted to compare the requirement of the chick for glutamic acid when fed "carbohydrate-free" and carbohydrate-containing diets, and to determine the effectiveness of other sources of nonessential nitrogen in meeting this requirement. Results showed that either L-glutamic acid or L-aspartic acid could serve as the major source of nonessential nitrogen when nonprotein energy was supplied by either glucose or soybean oil; however, in the absence of dietary carbohydrate the requirement increased. In the absence of dietary carbohydrate when nonprotein energy was supplied by soybean oil, diammonium citrate was less effective in promoting growth than L-glutamic acid or L-aspartic acid; DL-serine was still less effective, and DL-alanine and ammonium acetate almost completely failed to promote growth. The addition of carbohydrate to the diet markedly improved growth of chicks fed either diammonium citrate or ammonium acetate, but DL-alanine still failed to promote growth. Although DL-serine was more effective than DL-alanine, it was less effective than either L-glutamic acid, L-aspartic acid or diammonium citrate. Further studies showed that chicks fed a carbohydrate-containing diet in which L-alanine served as the major source of nonessential nitrogen grew at the same rate as chicks fed a similar diet containing L-aspartic acid, although slightly but significantly slower than chicks fed L-glutamic acid. The failure of chicks to grow when DL-alanine served as the major source of nonessential nitrogen in a carbohydrate-containing diet is due to the D-isomer.

Results of previous studies (1, 2) have shown that calories from fat can completely replace calories from carbohydrate in the diet of the chick without altering growth rate. Since dispensable amino acids are derived from carbohydrate the question arose as to whether chicks fed carbohydrate-free diets could synthesize them when given a source of nonessential nitrogen, or whether they must be provided preformed in the diet. Because glutamic acid plays a key role in the synthesis of other dispensable amino acids, initially, studies were conducted to compare the requirement of the chick for glutamic acid when fed carbohydrate-containing and carbohydrate-free diets. Subsequently, experiments were conducted to compare the effectiveness of other sources of nonessential nitrogen in meeting the requirement for synthesis of dispensable amino acids.

EXPERIMENTAL

Five experiments were conducted in which duplicate groups of 10 male Dominant White × White Plymouth Rock chicks were fed carbohydrate-free or carbohydrate-containing diets in which nonprotein energy was supplied by soybean oil or a mixture of glucose and soybean oil, respectively.

The semipurified diets used in all experiments contained the constant dietary ingredients shown in table 1. The amino acid mixture was patterned after that of Dean and Scott (3) with the exception that glutamic acid was deleted from the mixture. When DL- forms of amino acids were used, the amino acids were included at twice the level recommended for the

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TABLE 1

Composition of diets (exp. 1)

	Carbohydrate	
	Containing	"Free"
	g	*g*
Constants		
Amino acid mix [1]	14.60	
Soybean oil	15.00	
Cellulose [2]	5.00	
Vitamin mix [3]	0.51	
Mineral mix [4]	9.25	
Antioxidant [5]	0.025	
Chromium oxide	0.30	
Variables		
Glutamic acid	8.14	8.14
Cornstarch	47.18	—
Cellulose	—	4.66
Soybean oil	—	20.75

[1] Amino acid mixture supplied: (in grams) L-lysine·HCl, 1.40; L-leucine, 1.20; DL-isoleucine, 1.60; DL-valine, 1.64; DL-methionine, 0.55; L-arginine·HCl, 1.33; L-histidine·HCl·H_2O, 0.41; DL-threonine, 1.30; L-tryptophan, 0.225; L-tyrosine, 0.63; L-cystine, 0.35; glycine, 1.60; L-proline, 1.00; and DL-phenylalanine, 1.36.

[2] Solka Floc SW-40-A, Brown Forest Products Limited, Montreal, Quebec, Canada.

[3] Vitamin mixture supplied: (in milligrams per 100 g diet) thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.04; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 220; inositol, 10; *p*-aminobenzoic acid, 0.2; ascorbic acid, 25; vitamin A palmitate, 1000 USP units; vitamin D_3, 150 ICU; vitamin E acetate, 3.31 IU; and chlortetracycline, 1.

[4] Mineral mixture supplied: (in milligrams per 100 g diet) $CaHPO_4 \cdot 2H_2O$, 4670; $CaCO_3$, 750; $KHCO_3$, 1900; $NaHCO_3$, 1600; $MnSO_4 \cdot H_2O$, 33; $FeSO_4 \cdot 7H_2O$, 33; $MgSO_4$, 250; KI, 0.26; $CuSO_4 \cdot 5H_2O$, 1.67; $ZnCO_3$, 11.5; $CoCl_2 \cdot 6H_2O$, 0.17; $NaMoO_4 \cdot 2H_2O$, 0.76; and Na_2SeO_3, 0.022.

[5] Ethoxyquin.

L- form, except for methionine. The mineral mixture used was low in chloride, the chloride requirement being met by the hydrochlorides of the essential amino acids. Nonessential nitrogen was supplied by glutamic acid or by an isonitrogenous amount of some other substance or mixture of substances.

Carbohydrate-containing diets were formulated by the addition of starch to the constant dietary ingredients. Their carbohydrate-free counterparts were formulated by replacing starch isocalorically with soybean oil, using the values 4.21 and 9.21 kcal/g for the metabolizable energy content of starch and soybean oil, respectively. Extra cellulose was added to improve the texture of the carbohydrate-free diet. Because the carbohydrate-free diets do not total to 100, their content of nonessential nitrogen will be referred to as the level present in the carbohydrate-containing diets from which they were derived.

All diets were high in energy. Calculations indicated that they contained about 24.7 kcal metabolizable energy/g protein in experiments 1, 3, 4 and 5, and 23.0 kcal metabolizable energy/g protein in experiment 2. In some experiments, for comparative purposes a mixture containing 93.8 parts soybean protein, 3.6 parts methionine and 2.7 parts glycine was substituted isonitrogenously for the mixture of indispensable and dispensable amino acids.

The chicks were fed to 7 days of age the carbohydrate-free diet containing 24.7 kcal metabolizable energy/g protein in which protein was supplied by the mixture of soybean protein, methionine and glycine, and nonprotein energy by soybean oil. They were then allotted on the basis of body weight to the experimental groups and fed the experimental diets to 14 days of age. The chicks were maintained in electrically heated, thermostatically controlled, battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum. Data on growth and feed consumption were obtained after 7 days on experiment. Feed wastage was determined daily.

RESULTS

Experiment 1 was designed to compare the requirement of the chick for glutamic acid when fed carbohydrate-containing and carbohydrate-free diets. Three levels of glutamic acid were fed, 8.14, 4.07 and 0%. As the level of glutamic acid was reduced, an isonitrogenous amount of ammonium acetate was added to maintain the calorie-to-nitrogen ratio constant.

Results summarized in table 2 show that in the absence of glutamic acid when nonessential nitrogen was supplied entirely by ammonium acetate, chicks fed the carbohydrate-containing diet grew, but at a significantly slower ($P < 0.05$) rate than when glutamic acid was present, whereas chicks fed comparable carbohydrate-free diets failed to grow almost completely in the absence of glutamic acid. The data indicate the requirement of the

TABLE 2

Effect of level of glutamic acid on growth of chicks fed diets with and without carbohydrate (exp. 1)

Source of nonessential N		Wt gain		Nitrogen retention [1]	
		Carbohydrate		Carbohydrate	
Glutamic acid	NH_4 acetate	Containing	"Free"	Containing	"Free"
%	%	*g/day*	*g/day*	%	%
8.14	—	12.0 [2,e]	9.6 [c,d]	84 [c,d]	88 [d]
4.07	2.24	11.0 [d,e]	7.2 [b]	81 [b,c]	77 [b]
—	4.48	8.8 [c]	0.8 [a]	79 [b,c]	27 [a]
Soybean protein [3]	—	10.0 [c,d]	10.3 [d]	83 [c,d]	80 [b,c]

[1] (Gain in carcass protein, g/protein consumed, g) × 100.
[2] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.
[3] Dietary nitrogen supplied by a mixture containing 93.8 parts soybean protein, 3.6 parts methionine and 2.7 parts glycine in amounts to provide 16.9% protein.

chick for glutamic acid to be not greater than 4.07% when it is fed the carbohydrate-containing diet, and between 4.07 and 8.14% when it is fed the carbohydrate-free diet.

In experiment 2, carbohydrate-free diets containing graded levels of glutamic acid ranging from zero to 10% were fed. Results summarized in table 3 show that as in the preceding experiment, in the absence of carbohydrate and glutamic acid, when nonessential nitrogen was supplied by ammonium acetate chicks failed to grow. Analysis of variance and application of Duncan's multiple range test (4) to the data showed that chicks fed the diet containing 6% glutamic acid grew faster and consumed significantly ($P < 0.05$) more feed than chicks receiving 4% glutamic acid. These results indicate the glutamic acid requirement to be approximately 6% when chicks are fed carbohydrate-free diets.

TABLE 3

Effect of level of glutamic acid on growth of chicks fed "carbohydrate-free" diets (exp. 2)

Source of nonessential N		Wt gain	Feed consumption
Glutamic acid	NH_4 acetate		
%	%	*g/day*	*g/day*
10	—	8.4 [1,c]	9.8 [c]
8	1.05	8.6 [c]	9.8 [c]
6	2.09	8.3 [c]	9.8 [c]
4	3.14	6.4 [b]	8.4 [b]
0	5.23	1.0 [a]	4.3 [a]
Soybean protein [2]	—	9.9 [d]	11.1 [d]

[1] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.
[2] Dietary nitrogen supplied by a mixture containing 93.8 parts soybean protein, 3.6 parts methionine and 2.7 parts glycine in amounts to provide 18.1% protein.

To determine the effectiveness of other sources of nonessential nitrogen in promoting growth of chicks fed carbohydrate-free diets, duplicate groups of chicks were fed diets in which nonessential nitrogen was supplied by L-glutamic acid, L-aspartic acid, diammonium citrate, DL-serine, DL-alanine or ammonium acetate (exp. 3). The level fed was 6% except in the case of ammonium acetate which was fed at a level of 5.2%. All diets were maintained isonitrogenous by the addition of ammonium acetate.

Analysis of variance and application of Duncan's multiple range test (4) to the growth data (table 4) showed that L-aspartic acid was as effective as L-glutamic acid in meeting the requirement of the chick for nonessential nitrogen. Results showed, however, that diammonium citrate was significantly less effective ($P < 0.05$), whereas DL-serine and DL-alanine, like ammonium acetate, could not serve as the sole source of nonessential nitrogen for chicks fed carbohydrate-free diets.

The results of a similar experiment (exp. 4) comparing the effectiveness of L-glutamic acid, L-aspartic acid, diammonium citrate, DL-serine, DL-alanine and ammonium acetate in promoting growth of chicks fed carbohydrate-containing diets are also summarized in table 4. Because results of experiment 1 indicated the requirement of the chick for glutamic acid in the presence of dietary carbohy-

TABLE 4

Effect of source of nonessential nitrogen on growth and feed consumption when chicks are fed "carbohydrate-free" and carbohydrate-containing diets

Source of nonessential N	Exp. 3 "Carbohydrate-free"		Exp. 4 Carbohydrate-containing	
	Wt gain	Feed consumed	Wt gain	Feed consumed
	g/day	*g/day*	*g/day*	*g/day*
L-Glutamic acid [1]	8.1 [2,c]	10.3 [d]	9.8 [c]	18.1 [b]
L-Aspartic acid [1]	8.1 [c]	9.9 [d]	11.4 [c]	19.2 [b]
$(NH_4)_2$ citrate [1]	5.4 [b]	8.6 [c]	9.8 [c]	18.6 [b]
DL-Serine [1]	2.8 [a]	4.8 [b]	5.0 [b]	11.0 [a]
DL-Alanine [1]	1.4 [a]	3.9 [a]	1.5 [a]	9.0 [a]
NH_4 acetate [3]	1.3 [a]	5.1 [b]	9.6 [c]	18.9 [b]
Soybean protein [4]	—	—	9.8 [c]	19.2 [b]

[1] Level of incorporation was 6% in experiment 3 and 4% in experiment 4. Diets maintained isonitrogenous by the addition of ammonium acetate.
[2] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.
[3] Level of incorporation was 5.19% in experiment 3 and 4.19% in experiment 4.
[4] See table 2, footnote 2.

drate to be not more than 4%, all sources were incorporated at this level. Ammonium acetate was added to maintain the same level of nitrogen in all diets.

Analysis of variance and application of Duncan's multiple range test (4) to the data on growth and feed consumption indicated that glutamic acid, aspartic acid, diammonium citrate and ammonium acetate were equally effective in promoting growth and feed consumption of chicks fed carbohydrate-containing diets and significantly ($P < 0.05$) more effective than either DL-serine or DL-alanine. The observation that ammonium acetate was as effective as glutamic acid in promoting growth of chicks fed carbohydrate-containing diets was in contrast to results of experiment 1, which showed that ammonium acetate was less effective than a mixture of glutamic acid and ammonium acetate. The reason for this discrepancy was not apparent.

Comparison of the results of experiments 3 and 4 (table 4) also indicates that DL-serine is more effective in promoting growth of chicks fed carbohydrate-containing diets than carbohydrate-free diets. Whether this is due to the source of energy, or to the difference in level fed (4 against 6%) awaits further study.

To determine whether the growth-depressing effect of DL-alanine was due to the racemic mixture fed or to the level in the diet, or both, chicks were fed carbohydrate-containing diets in which nonessential nitrogen was supplied by isonitrogenous amounts of L-alanine, DL-alanine or a mixture of DL-alanine and ammonium acetate supplying one-half as much DL-alanine (exp. 5). For comparative purposes chicks were also fed diets in which nonessential nitrogen was supplied by L-glutamic acid, L-aspartic acid, asparagine and ammonium acetate. Data summarized in table 5 show that, as in experiment 4, chicks consumed less feed and failed to grow when DL-alanine served as the major source of nonessential nitrogen. Analysis of variance and application of Duncan's multiple range test (4) to the data on growth and feed consumption showed that chicks fed L-alanine as the major source of nonessential nitrogen grew at the same rate and consumed similar amounts of feed as chicks fed a comparable diet containing aspartic acid. In this experiment, both growth and feed consumption of chicks fed L-alanine were slightly but significantly lower than chicks fed L-glutamic acid. Thus, it can be concluded that the failure of chicks to grow when DL-alanine serves as the major source of nonessential nitrogen in a carbohydrate-containing diet is due to the D-isomer. The results also show that replacing 50% of the DL-alanine by an isonitrogenous amount of ammonium acetate reduced the growth-depressing effect, but chicks still grew less rapidly and con-

TABLE 5

Effect of source of nonessential nitrogen on growth and feed consumption when chicks are fed carbohydrate-containing diets (exp. 5)

Source of nonessential N		Wt gain	Feed consumption
Variable	Level		
	%	*g/day*	*g/day*
L-Glutamic acid	7.98	11.3 [1,e]	18.7 [d,e]
L-Aspartic acid	7.22	10.8 [d,e]	18.0 [c,d]
Asparagine	3.58	10.0 [d,e]	18.0 [c,d]
DL-Alanine	4.83	0.9 [a]	6.4 [a]
DL-Alanine [2]	2.42	4.7 [b]	11.0 [b]
L-Alanine	4.83	9.2 [c,d]	16.2 [c]
NH_4 acetate	4.18	7.8 [c]	16.2 [c]
Soybean protein [3]	—	11.6 [e]	20.3 [e]

[1] Values are averages of duplicate groups. Values without a common letter in their superscript are significantly different.
[2] Ammonium acetate (2.09%) added to maintain diet isonitrogenous.
[3] See table 2, footnote 2.

sumed less feed than chicks receiving ammonium acetate as the major source of nonessential nitrogen.

That the growth depressing effect of DL-alanine cannot be overcome by deleting other D-amino acids from the diet was shown in subsequent studies [2] in which DL-alanine was added to diets in which nitrogen was supplied by a mixture of soybean protein, glycine and methionine. Results showed that the addition of 2.42% DL-alanine to the diet depressed growth by 33%.

DISCUSSION

The observation that chicks fed carbohydrate-free diets were unable to use ammonium acetate as a source of nonessential nitrogen, but could use ammonium citrate, suggests that the effectiveness of ammonium salts as a source of nonessential nitrogen depends on whether the anion is glucogenic or ketogenic. Because nonessential amino acids are glucogenic in origin, this finding supports our previous observation (5) that the ability of the chick to synthesize carbohydrate precursors from triglycerides is limited.

Studies showed that the requirement of chicks for glutamic acid was increased when carbohydrate was deleted from the diet, thus giving additional support to the concept that the ability of the chick to synthesize carbohydrate from triglyceride is limited. Since L-aspartic acid was just as effective as L-glutamic acid in meeting this requirement it can be concluded that the requirement is not specific. Whether L-alanine or L-serine would be as effective as L-aspartic acid and L-glutamic acid in promoting growth of chicks fed carbohydrate-free diets was not determined.

The response of chicks fed carbohydrate-containing diets to ammonium acetate as a source of nonessential nitrogen was variable. In experiment 4, it was found to be as effective as L-glutamic acid, L-aspartic acid and diammonium citrate, whereas in experiments 1 and 5, it was approximately 70% as effective as L-glutamic acid. The reason for this discrepancy was not apparent.

Results of experiment 4 (table 4) showed that with diets containing carbohydrate, diammonium citrate was as effective as L-glutamic acid in meeting the requirement of the chick for nonessential nitrogen. This observation is in contrast to results recently reported by Scott et al. (6) who found that the isonitrogenous substitution of diammonium citrate for 10% L-glutamic acid reduced weight by 20%.

The observation that D-alanine, when incorporated in the diet at a level of 1.21%, reduced feed intake and growth is in agreement with results reported by Adkins et al. (7). They found that the addition of 1.25% D-alanine depressed growth by about 50%. More recently Sugahara et al. (8) reported that D-alanine at the 1% level caused slight growth retardation.

Whether the decreased effectiveness of DL-serine as a source of nonessential nitrogen is due to the D-isomer was not determined. Sugahara et al. (8) reported no growth depression when 1.90% D-serine replaced 1.90% L-serine in semipurified diets containing crystalline amino acids as the sole source of nitrogen.

Greene et al. (9) observed growth depression when a mixture containing 2.16% DL-alanine, 2.28% L-aspartic acid and 4.40% DL-serine was added to an amino acid diet containing 12.85% glutamic acid. They suggested that the

[2] Unpublished data.

growth-depressing effect might be due to an unfavorable ratio of essential amino acids to nonprotein nitrogen in the ration. Results of the present studies suggest that DL-alanine and DL-serine may have contributed to the growth depression.

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Relationship of Maternal Dietary Zinc during Gestation and Lactation to Development and Zinc, Iron and Copper Content of the Postnatal Rat [1,2]

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ABSTRACT High levels (0.2 and 0.5%) of zinc were fed to adult female rats beginning at zero-day age of the fetus and continued to day 14 of lactation to study the development and iron, copper, and zinc status of zero- and 14-day-old postnatal rats. The results were compared with rats fed a basal diet containing 9 ppm zinc. Growth reduction, in terms of dry matter content, was characteristic of the zero- and 14-day-old young from mothers fed 0.5% zinc. Though no anatomical malformations were observed, the incidence of stillbirths was high in the 0.5% zinc group. Young from mothers fed 0.2% zinc were larger in body size than those from mothers fed the basal diet. Total zinc and concentration of zinc were elevated in zero- and 14-day-old young by both high zinc diets. Young from mothers fed 0.5% zinc contained higher levels of zinc than those from mothers fed 0.2% zinc. Bodies of newborn young from mothers fed 0.5% zinc contained significantly less total iron and concentration of iron, whereas the liver contained significantly more iron. At 14 days of age the whole animal and body of young from mothers fed 0.5% zinc contained significantly less total iron and concentration of iron. Liver iron in these young was lower than found in the liver of newborn, and total iron and concentration of iron in the liver were similar for all groups. Total copper and concentration of copper in the whole animal and body of the newborn rats were not altered due to treatment. Total copper and concentration of copper in the liver were significantly lower only in the newborn from mothers fed 0.5% zinc. After 14 days, total copper and concentration of copper were significantly lower in the whole animal, liver and body of young from mothers fed either zinc diet than in young from mothers fed the basal diet. In general, a larger reduction in copper occurred in the young from the 0.5% zinc treatment. Mothers fed 0.2% zinc gained more weight during gestation. Dietary treatments had no effect on body weights during lactation. Livers of maternal animals fed excess zinc contained elevated zinc, and reductions in both iron and copper.

The effect of excess (0.2 and 0.4%) zinc in the diet of the female rat on development and zinc, iron, and copper content of the fetal rat has been reported (1). Variable degrees of resorption and retardation of fetal development, but no external anatomical malformations, were observed in 15-to 20-day-old fetuses from mothers fed 0.4% zinc. The type of change and its severity depended upon the length of time mothers were fed 0.4% zinc. None of the manifestations was noted in rat fetuses from mothers fed 0.2% zinc. Concomitant with an elevation of the content of zinc in the fetuses from mothers fed 0.4% zinc were reductions in iron and copper levels similar to those reported (2–5) for the weanling and adult rat fed excess zinc. No elevation of zinc or change in iron was found in fetuses from mothers fed 0.2% zinc, but the copper content was reduced. The present investigation was designed to study the effect of excess zinc in the diet of the rat during gestation and lactation on development and zinc, iron and copper contents of the postnatal rat.

EXPERIMENTAL PROCEDURES

Nulliparous albino rats,[4] ranging in weight from 200 to 275 g, were individual-

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[4] CFE strain of Sprague-Dawley rats obtained from Carworth, New City, N. Y.

ly housed in galvanized wire cages and received feed and distilled water ad libitum. The composition of the basal diet was as follows: (in percent) casein,[5] 20; sucrose, 62; cellulose,[6] 3; corn oil, 10; salt mixture,[7] 4; and vitamin mixture,[8] 1. Excess zinc diets were made by the incorporation of either 0.2 or 0.5% zinc as zinc oxide into the basal diet. Zinc content of the basal diet was 9 ppm.

Mating was performed as previously described (6). The day sperm were found in the vaginal smear was designated as day 1 of gestation, and rats were randomly assigned, 10 to each diet, to either a basal, 0.2% zinc, or 0.5% zinc diet. Near the end of pregnancy, animals were observed frequently, and, in most cases, pups were detected shortly after birth and before they had suckled. Four pups from each litter were randomly selected and killed. Livers were excised and pooled, and the bodies [9] were pooled for analyses. Milk was removed from the stomachs of pups that had suckled. The remaining young in the litter were reduced to six in number and returned to their mothers, which were continued on their respective diets during lactation. At day 14 of lactation, mothers and pups were killed. Dry weights of liver, body and whole animal of the six pups were determined, and tissues of three pups were randomly selected for mineral analyses. Maternal livers were excised and rinsed with distilled water. Livers of pups were removed and pooled for analyses; bodies were analyzed separately. Therefore, the value for the whole animal [10] is the mathematical sum of the determined values for body and liver.

All specimens were dried to a constant weight at 100°. With the exception of 14-day-old bodies, specimens were wet digested with nitric and sulfuric acids. The 14-day-old bodies were ashed at 500° for 24 hours. Ash was dissolved in 5 ml of 30% (v/v) hydrochloric acid, and made to the appropriate volume with distilled water. Samples were analyzed for zinc (7), iron, using the 1,10-phenanthroline method (8), and copper (9). Data were analyzed statistically by analysis of variance and Duncan's (10) multiple range test.

RESULTS

Data illustrating the effect of excess dietary zinc during gestation and lactation on litter size and dry weight of whole animal, liver, and body of postnatal rats at birth (zero-day age) and 14 days of age are presented in table 1. Values were not included for a control mother that had only four pups and for two animals fed 0.5% zinc that had all stillborn animals.

Number of viable young per litter was not different between treatments. No external anatomical malformations were observed. In addition to the stillborn animals found in the rats fed 0.5% zinc, four stillborn animals were born to mothers fed 0.2% zinc. Edema was a characteristic feature of the stillborn animals from mothers fed 0.5% zinc, but not for those from mothers fed 0.2% zinc.

Dry weights of the whole animal and body of zero- and 14-day-old postnatal young from mothers fed 0.5% zinc were significantly less than those from mothers fed the basal or 0.2% zinc diets. Dry weights of the whole animal and body of zero-day-old young from mothers fed 0.2% zinc were significantly greater than young from mothers fed the basal diet. The average weight of the 14-day-old animals from mothers fed 0.2% zinc was larger than those from mothers fed the basal diet, but the difference was not significant. No significant difference in liver dry weight was found between groups at birth. At 14 days, however, the liver dry weight of young from mothers fed 0.5% zinc was significantly less than that of young from mothers fed the basal or 0.2% zinc diets.

Data showing the total amount (micrograms) and concentration (parts per mil-

[5] Vitamin-free casein, General Biochemicals Inc., Chagrin Falls, Ohio.

[6] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[7] Jones, J. H., and C. Foster 1942 J. Nutr., 24: 245 (obtained from Nutritional Biochemicals Corporation, Cleveland).

[8] Vitamins in cornstarch, amount per kilogram diet: vitamin A, 20,000 IU; vitamin D, 2200 IU; (in milligrams) ascorbic acid, 1017; vitamin E as α-tocopheryl acetate, 485; inositol, 110; choline dihydrogen citrate, 3715; menadione, 49.6; *p*-aminobenzoic acid, 110; niacin, 99.2; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 29.7 μg (obtained from General Biochemicals Inc., Chagrin Falls).

[9] In this report, the term body is used to mean the whole animal minus the liver. Whole-animal values are the mathematical sum of the determined values for liver and body.

[10] See footnote 9.

TABLE 1

Maternal weight, maternal feed intake, length of gestation period, number of young born, and dry weight of whole animal, liver, and body of young at zero day and 14 days of age

Diet	Maternal wt			Daily feed intake		Days of gestation	Viable young per litter	Dry wt		
	Initial	Day 21 of gestation	Day 14 of lactation	Gestation	Lactation			Liver	Body	Whole animal
	g	*g*	*g*	*g*	*g*	*days*		*mg*	*mg*	*mg*
									Newborn [1]	
Basal (9) [2]	236 ± 2 [3]	343 ± 6	252 ± 9	16.4 ± 0.7	22.9 ± 0.3	22.4 ± 1.1	12.3 ± 0.5	56.2 ± 2.9	708 ± 21 [a 4,5]	764 ± 22 [a 4,5]
0.2% Zn (10)	233 ± 2	357 ± 3	262 ± 4	17.4 ± 0.5	23.1 ± 0.1	23.2 ± 0.7	11.6 ± 0.5	56.1 ± 1.7	797 ± 24 [b]	857 ± 23 [b]
0.5% Zn (8)	227 ± 5	339 ± 1	252 ± 7	16.7 ± 0.7	23.7 ± 0.4	23.7 ± 0.6	12.4 ± 0.4	47.4 ± 2.2	632 ± 18 [c 5]	687 ± 21 [c 5]
									14-day old [6]	
Basal (9)								235.3 ± 10.6 [a 4]	8650 ± 430 [a 7]	8885 ± 443 [a 7]
0.2% Zn (10)								240.7 ± 8.4 [a]	9230 ± 210 [a]	9474 ± 215 [a]
0.5% Zn (8)								200.2 ± 9.0 [b 5]	6890 ± 410 [b]	7088 ± 418 [b]

[1] Average values of four animals per litter per treatment.
[2] Number in parentheses indicates number of maternal animals.
[3] Mean and SE.
[4] Values followed by different letters differ significantly at $P < 0.05$.
[5] Significantly different from the 0.2% Zn treatment at $P < 0.01$.
[6] Average values of six animals per litter per treatment.
[7] Values followed by different letters differ significantly at $P < 0.01$.

lion) on a dry weight basis of zinc, iron, and copper in the whole animal, liver, and body of zero- and 14-day-old young from mothers fed the various diets are tabulated in tables 2, 3, and 4.

Total zinc and concentration of zinc in the whole animal, liver, and body of newborn young from mothers fed either high zinc diet were significantly higher than those from mothers fed the basal diet. The whole animal and body of newborn animals from mothers fed 0.5% zinc contained a significantly higher zinc concentration than those from mothers fed 0.2% zinc. Total zinc, however, was not higher. No significant difference in zinc content was found in the livers of young from mothers fed diets containing 0.2 or 0.5% zinc.

At 14 days, the whole animal, liver, and body of pups from mothers fed 0.5% zinc contained significantly more total zinc and a higher concentration of zinc than pups from mothers fed the basal diet. Whole animals and bodies of young from mothers fed 0.2% zinc contained higher total zinc and concentration of zinc than young from mothers fed the basal diet; only total zinc was significantly different. Livers of young from mothers fed 0.2% zinc contained a significantly higher concentration of zinc, but not total zinc, than the livers of young from mothers fed the basal diet. Livers of pups from mothers fed 0.5% zinc contained significantly more total zinc and concentration of zinc than those of mothers fed 0.2% zinc.

No significant differences were found between treatments in total iron and concentration of iron in the whole animal of newborn rats; however, the trend did indicate lower values in animals from mothers fed zinc. Bodies of young from mothers fed 0.5% zinc contained significantly less total iron and concentration of iron than those from the control group, and significantly less total iron than those of young from mothers fed 0.2% zinc. In contrast, the livers of newborn from mothers fed either high zinc diet contained significantly more total iron and concentration of iron than the livers of young from mothers fed the basal diet. Iron concentration was significantly less in the bodies of young from the 0.2% zinc group

TABLE 2

Zinc content on dry weight basis of liver, body and whole animal of newborn and 14-day-old rats

Diet	Liver		Body		Whole animal	
	Total	Conc	Total	Conc	Total	Conc
	µg	*ppm*	*µg*	*ppm*	*µg*	*ppm*
			Newborn [1]			
Basal	17.9 ± 1.9 [2 a 3]	319.0 ± 29.1 [a 3]	45.5 ± 1.9 [a 3]	65.4 ± 3.2 [a 3]	62.6 ± 1.9 [a 3]	82.8 ± 2.5 [a 3]
0.2% Zn	40.3 ± 4.2 [b]	718.7 ± 64.5 [b]	72.2 ± 5.8 [b]	89.6 ± 6.9 [b]	111.6 ± 6.1 [b]	128.1 ± 5.2 [b]
0.5% Zn	40.0 ± 2.9 [b]	842.9 ± 54.6 [b]	80.6 ± 5.1 [b]	127.3 ± 7.3 [c]	120.6 ± 7.0 [b]	176.9 ± 8.9 [c]
			14-day old [4]			
Basal	40.1 ± 3.9 [a 5]	180.4 ± 24.3 [a]	350.0 ± 22.0 [a 5]	42.1 ± 2.4 [a 3]	390.4 ± 21.3 [a 3]	43.7 ± 2.2 [a 3]
0.2% Zn	82.7 ± 6.4 [a]	348.4 ± 30.2 [b]	494.0 ± 25.5 [b]	54.0 ± 3.4 [a]	577.0 ± 27.0 [b]	61.4 ± 3.6 [a]
0.5% Zn	102.1 ± 10.9 [b]	499.9 ± 36.1 [c]	607.9 ± 71.4 [b 6]	87.8 ± 8.6 [b]	710.1 ± 73.8 [b]	101.1 ± 9.7 [b]

[1] Average values of four animals per litter per treatment.
[2] Mean and SE.
[3] Means followed by different letters differ significantly by $P < 0.01$.
[4] Average values of three animals per litter per treatment.
[5] Means followed by different letters differ significantly by $P < 0.05$.
[6] Significantly different from basal treatment at $P < 0.01$.

TABLE 3

Iron content on dry weight basis of liver, body and whole animal of newborn and 14-day-old rats

Diet	Liver		Body		Whole animal	
	Total	Conc	Total	Conc	Total	Conc
	μg	*ppm*	*μg*	*ppm*	*μg*	*ppm*
			Newborn [1]			
Basal	16.3 ± 2.1 [2,a,3]	300.1 ± 38.6 [a,4]	203.0 ± 26.7 [a,4]	284.6 ± 32.5 [a,4]	218.3 ± 27.3	284.3 ± 30.7
0.2% Zn	42.1 ± 7.7 [b]	764.6 ± 138.2 [b,5]	157.9 ± 11.5 [a]	197.6 ± 16.5 [b]	204.9 ± 11.7	238.5 ± 15.7
0.5% Zn	29.0 ± 3.9 [b]	629.2 ± 94.8 [b]	127.0 ± 16.3 [b]	199.9 ± 26.0 [b]	156.4 ± 16.3	230.7 ± 24.2
			14 day old [6]			
Basal	47.5 ± 7.9	199.5 ± 26.2	609.0 ± 23.7 [a,3]	71.3 ± 2.1 [a,4]	657.0 ± 29.2 [a,3]	74.5 ± 2.2 [a,4]
0.2% Zn	56.3 ± 13.1	232.2 ± 49.4	531.5 ± 24.6 [a]	57.8 ± 2.8 [b]	596.6 ± 33.2 [a]	63.1 ± 3.6 [b]
0.5% Zn	40.0 ± 5.1	196.3 ± 22.9	372.7 ± 39.8 [b]	58.1 ± 6.1 [b]	400.1 ± 40.7 [b]	57.0 ± 4.2 [b,5]

[1] See footnote 1, table 2.
[2] Mean and SE.
[3] Means followed by different letters differ significantly by $P < 0.01$.
[4] Means followed by different letters differ significantly by $P < 0.05$.
[5] Significantly different from basal treatment at $P < 0.01$.
[6] See footnote 4, table 2.

TABLE 4

Copper content on dry weight basis of liver, body and whole animal of newborn and 14-day-old rats

Diet	Liver		Body		Whole animal	
	Total	Conc	Total	Conc	Total	Conc
	μg	*ppm*	*μg*	*ppm*	*μg*	*ppm*
			Newborn [1]			
Basal	2.7 ± 0.4 [2,a,3]	48.3 ± 6.3 [a,3]	8.6 ± 0.5	12.4 ± 0.8	11.5 ± 0.3	15.5 ± 0.6
0.2% Zn	2.4 ± 0.5 [a]	42.7 ± 7.8 [a]	10.9 ± 1.1	13.6 ± 1.3	13.0 ± 0.9	15.0 ± 1.0
0.5% Zn	1.0 ± 0.2 [b,4]	21.7 ± 3.6 [b,4]	8.9 ± 1.2	14.2 ± 1.8	10.0 ± 1.1	14.7 ± 1.6
			14-day old [5]			
Basal	28.9 ± 2.3 [a,6]	125.9 ± 11.8 [a,3]	54.0 ± 4.1 [a,6]	6.3 ± 0.5 [a,3]	82.9 ± 4.0 [a,6]	9.5 ± 0.5 [a,6]
0.2% Zn	11.8 ± 1.9 [b]	50.4 ± 9.0 [b,4]	38.6 ± 3.8 [b]	4.2 ± 0.4 [b,4]	50.5 ± 4.6 [b]	5.3 ± 0.5 [b]
0.5% Zn	4.1 ± 0.4 [c]	20.1 ± 1.6 [c,4]	31.7 ± 1.6 [b]	4.9 ± 0.4 [b]	35.7 ± 1.6 [c]	5.3 ± 0.4 [b]

[1] See footnote 1, table 2.
[2] Mean and SE.
[3] Means followed by different letters differ significantly by $P < 0.05$.
[4] Significantly different from basal treatment at $P < 0.01$.
[5] See footnote 4, table 2.
[6] Means followed by different letters differ significantly by $P < 0.01$.

compared with those from the basal group. Total iron was also less in the body, but the difference was insignificant.

At 14 days, the whole animal and body of young from the 0.5% zinc treatment contained significantly less total iron than those of animals from mothers fed 0.2% zinc or basal diet. No difference was found between the 0.2% zinc and basal groups. Based on concentration, the whole animal and body of postnatal animals from both experimental treatments contained significantly less iron than those in the basal group. Instead of a high liver iron as observed for newborn animals in the experimental groups, total iron and concentration of iron in the liver of 14-day-old young were similar for all groups.

No significant changes were found between treatments in total copper and concentration of copper in the whole animal and body of the newborn rat. Total copper and concentration of copper in the liver of young from the 0.5% zinc treatment were significantly less than in the liver of young from the basal or 0.2% zinc diets. No difference in liver copper was found between the newborn young from mothers fed 0.2% zinc and mothers fed the basal diet.

Total copper and concentration of copper were significantly lower in the whole animal, liver, and body of 14-day-old young from mothers fed either zinc diet compared with those from mothers fed the basal diet. The whole animal from the 0.5% zinc group contained significantly less total copper and the liver significantly less total copper and concentration of copper than the whole animal and liver from the 0.2% zinc group.

Changes in zinc and iron content in young between zero and 14 days of age were an increase in total and a decrease in concentration, irrespective of treatment. The largest increase in liver total iron occurred in young from mothers fed the basal diet. Change in liver copper content between zero and 14 days varied between treatments. Total copper increased about 10-fold in the livers of young from the basal treatment, but only approximately 5- and 4-fold in the livers of young from the 0.2 and 0.5% zinc treatments, respectively. A more striking observation was the absolute increase in liver copper. There was a change from 1.0 to 4.1 μg in the young from mothers fed 0.5% zinc compared with 2.7 to 28.9 μg in young from mothers fed the basal diet. Concentration of liver copper increased markedly in young from mothers fed the basal diet, but remained about the same in the liver of young from mothers fed the excess zinc diets. Total copper increased and concentration of copper decreased in the body and whole animal between zero and 14 days of age.

Data illustrating body weight, daily feed intake, and length of gestation period of maternal animals fed the various dietary regimens are tabulated in table 1. Values showing the amount of zinc, iron, and copper expressed as total (micrograms) and concentration (parts per million) on a dry weight basis in the maternal liver are shown in table 5.

No significant difference in maternal body weight was found between treatments at parturition and day 14 of lactation, although weight of mothers fed 0.2% zinc was consistently higher at these times. Also, the young of these mothers were larger at birth and 14 days than those in the other groups. No loss in body weight of the mothers occurred during lactation. Slight variations between groups occurred in daily feed intake of the mothers; however, the differences were not significant. Though the average length of gestation was about 1 day longer for the mothers fed the high zinc diets, the difference was insignificant.

Livers of maternal animals in both experimental groups contained a significantly higher total zinc and concentration of zinc than livers of animals in the basal group. Total zinc and concentration of zinc were significantly higher in livers of animals fed 0.5% zinc than in the liver of animals fed 0.2% zinc. Significant reductions occurred in total iron and copper and in concentration of these minerals in the liver of animals fed the diets containing additional zinc.

DISCUSSION

Nourishing the rat during development, from the embryo to weanling animal, is accomplished by intrauterine and mam-

TABLE 5

Dry weight and zinc, iron and copper content on dry weight basis of livers of maternal animals

Diet	Dry wt	Zinc		Iron		Copper	
		Total	Conc	Total	Conc	Total	Conc
	g	*μg*	*ppm*	*μg*	*ppm*	*μg*	*ppm*
Basal (9) [1]	3.434 ± 0.288 [2]	108.8 ± 15.2 [a 3]	31.8 ± 3.4 [a 3]	1144.4 ± 189.2 [a 4]	400.2 ± 118.5 [a 4]	66.4 ± 4.0 [a 3]	20.5 ± 1.8 [a 4]
0.2% Zn (10)	3.509 ± 0.191	358.0 ± 51.0 [b]	103.4 ± 14.5 [b]	636.0 ± 70.9 [b 5]	185.3 ± 22.5 [b 5]	52.0 ± 3.4 [b]	15.3 ± 1.4 [b]
0.5% Zn (8)	3.409 ± 0.213	766.0 ± 62.8 [c]	234.6 ± 63.3 [c]	662.9 ± 63.3 [b]	200.1 ± 20.4 [b]	43.8 ± 2.2 [b]	13.2 ± 0.8 [b 5]

[1] Number in parentheses indicates number of animals in group.
[2] Mean and SE.
[3] Means followed by different letters differ significantly at $P < 0.01$.
[4] Means followed by different letters differ significantly at $P < 0.05$.
[5] Significantly less than basal treatment at $P < 0.01$.

mary transfer of nutrients, through degenerating cells of the uterine epithelium, maternal blood via the allantoic and yolk-sac placentas, and milk. The kind and amount of nutrients available to the prenatal and postnatal animal will neither be the same nor remain constant during gestation and lactation because the composition of epithelial cells, the nutrients transferred via the placentas, and the composition of milk are influenced by the nutritional status of the mother. It is recognized that changes in nutrient availability, owing to the nutritional status of the mother, during these stressful periods can cause subtle and marked anatomical and physiological alterations in the young.

Excess zinc (0.5%) in the diet of the maternal rat beginning at day 1 of pregnancy caused a diminution in body size (in terms of dry matter content) of the newborn and stillborn animals. No external anatomical malformations were evident. In previous work (1), the reduction in body size, the stillborn animals, and the lack of anatomical malformations were also observed for the 15- to 18-day-old fetus from mothers fed 0.4% zinc. Other than small body size, the 14-day-old postnatal rat from mothers fed 0.5% zinc did not develop additional change in their physical appearance. Of interest, zero- and 14-day-old young from mothers fed 0.2% zinc had a larger body size than young from mothers fed the basal diet. This observation would tend to indicate that the requirement for zinc during gestation and lactation is in excess of the 9 ppm of zinc in the basal diet.

Zinc content was higher in the newborn and 14-day-old animals from mothers fed either 0.2 or 0.5% zinc compared with young from mothers fed the basal diet. Although a high zinc content was noted in the 14-day-old young from mothers fed the diets containing excess zinc, there is apparently a limit in the amount of zinc that can be transferred across the mammary barrier. Miller et al. (11) reported that 1000 ppm dietary zinc gave the same concentration of zinc in bovine milk as did 2000 ppm. Irrespective of diet, total zinc was consistently higher in the 14-day-old young compared with the newborn. Spray and Widdowson (12) reported that

there was an increase in body zinc of the rat during growth, and that a high proportion of this zinc was associated with skin and hair. This apparent high requirement for zinc for synthesis of these epidermal structures is easily provided in milk, which is high in zinc (13).

At birth, a reduced iron content was the trend in the whole animal containing an elevated zinc content. There was a significant diminution in body iron but a significant increase in liver iron, and the value for whole-animal iron was the summation of these two levels. Similar results were found (1) for the 18-day-old fetus. High iron content in the liver with concomitant high zinc may be the result of reduced activity of liver xanthine oxidase, which is related to the release of iron from ferritin stores in the fetus (14). A reduction of the activity of xanthine oxidase was found (15) in the adult rat fed excess zinc.

A significant reduction in iron content due to excess zinc was noted in whole animal and body at day 14 of postnatal age. Although liver iron was higher in the newborn from mothers fed zinc, indicating an aberration in iron utilization, at 14 days liver iron was similar in all groups, which suggests utilization. This is difficult to explain if xanthine oxidase was being inactivated by zinc. Possibly other mechanisms are involved in iron mobilization; for example, ceruloplasmin. Ceruloplasmin has been proposed (16) as being involved in promoting iron saturation of transferrin and possibly other iron-containing proteins and enzymes, and consequently the overall iron turnover in the body. However, the reduced activity of ceruloplasmin in rats fed excess dietary zinc [11] (17) makes the suggestion of its involvement tenuous. It should be emphasized that since neither xanthine oxidase nor ceruloplasmin was completely inactivated in animals fed zinc, there exists the possibility of adequate activity to be effective in mobilizing iron. Finally, if the iron in the liver of the zero- and 14-day-old rats is in the form of ferritin, the observation of an elevated liver iron in the newborn and subsequent release during the 14 days of lactation in rats with an elevated zinc content would tend to contradict the hypothesis (18) that zinc interferes with iron incorporation and release from ferritin.

Other than the antagonism of zinc with iron and copper in tissues of the young during lactation, the low iron and copper contents found in the 14-day-old rat may be the result of less iron and copper in milk of mothers fed zinc. Reduced iron and copper content in the milk could be due to either zinc interfering with passage of the minerals through the mammary barrier or a reduction in the iron and copper stores in the mother. In addition, a reduction in the volume of milk could cause an overall diminution in daily intake of iron and copper.

Various factors can lower the volume of milk of the lactating animal. Pertinent to this study, Mueller and Cox (19) showed a reduction in milk volume by increasing the calcium content in the diet of the lactating rat. Miller et al. (11) reported no alteration in milk production of the lactating dairy cow fed 2000 ppm zinc. Reduction in the growth rate of young could be a sign of a reduction in milk volume. In this regard, the 14-day-old postnatal rat from mothers fed excess zinc contained less dry matter than those from mothers fed the basal diet. This difference may be a reflection of the small amount of dry matter at birth; however, the percentage increase in dry matter was 1063, 1005 and 922% for young from mothers fed basal, 0.2% zinc and 0.5% zinc diet, respectively. The small difference in increase in dry matter between animals fed the various diets does not appear sufficiently large to indicate an appreciable reduction in milk volume.

Direct evidence concerning the level of iron and copper in the milk of mothers consuming excess zinc is not available. Though rat milk is high in iron compared with cow milk and human milk (20), and the level is maintained relatively constant during lactation (21), it is not high enough to maintain the body iron concentration found at birth (12). The amount of copper in rat milk is similar to that of iron (20), but there is a progressive decrease in amount during lactation (21).

[11] Lee, D. D., and G. Matrone 1968 Restoration of serum ceruloplasmin synthesis in serum of rats with zinc induced copper deficiency. Federation Proc., 27: 482 (abstract).

Hence, even during normal lactation the content of iron and copper in rat milk is low, and may be insufficient to adequately meet the growth demands of the postnatal animal. Consequently, a factor that could reduce the level of these minerals in milk would affect the level in the tissues of the young, and would adversely affect metabolic activities.

Rat milk normally contains a relatively high concentration of zinc and adequately provides for the production of fur and growth of the animal. Zinc is increased in milk of the bovine (11, 22) and porcine (23) fed high levels of dietary zinc. This infers an elevation of zinc in the mammary tissue, and suggests the possibility of an interference with the normal metabolism of iron and copper in the gland and their transfer to milk.

Since mothers fed excess zinc show signs of iron and copper deficiency, it is suggested that a decreased supply of these minerals is available for transfer to the milk. No data were found in the literature demonstrating an adverse effect of an iron deficiency in the maternal animal on iron content of the milk. Beck (24) noted that the copper content of milk from cows and ewes grazing copper-deficient pastures was considerably below the content in milk of animals fed normal diets.

No loss in body weight was found in mothers during 14 days of lactation. In contrast, Spray (13) reported a decline of nearly 20% in the body weight of rats during the 15-day postparturition period.

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Dietary Fat and the Inhibition of Hepatic Lipogenesis in the Mouse [1]

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ABSTRACT We have examined the effects of dietary fat upon lipogenesis from 1-^{14}C-acetate by slices of mouse liver, under two sets of conditions: a) a high fat diet (15% corn oil) was fed for 1 to 5 days, following zero, 1 and 2 days of fasting; and b) corn oil, safflower oil, coconut oil, tricaprylin, tripalmitin, triolein, oleic acid and mineral oil (all at 10% of the diet) were fed for 3 days, with no prior fasting. Of the high fat diets only those high in linoleic acid suppressed fatty acid synthesis. Corn oil reduced fatty acid synthesis in nonfasted mice, and suppressed the usual "adaptive hyperlipogenesis" found after fasting and refeeding. From this work, and that of others, it is now clear that any alteration in hepatic lipogenesis induced by the inclusion of fat in the diet will depend not only on the fatty acid composition of the fat, but also on the level in the diet, the length of time it is fed, the animal species and age examined, and on whether the studies are conducted in vivo or in vitro.

Since the early studies of Boxer and Stetten (1) and Bernhard and Steinhauser (2), many workers have examined the effects of variations in diet upon subsequent hepatic lipogenesis, both in vivo and in vitro. Recently this work has concentrated upon two aspects: a) "meal-feeding" versus "nibbling," i.e., intermittent periods of fasting and feeding as opposed to feeding ad libitum (3–7); and b) the so-called "feedback inhibition" of synthetic pathways, particularly of fatty acids (7–10) and of cholesterol (11–14).[4]

Chaikoff and his colleagues (10, 15, 16) have examined various aspects of "feedback" inhibition of fatty acid synthesis, i.e., the depression of synthesis of long-chain fatty acids following the feeding of a high fat diet. Their work (10) indicated that various fats, corn oil, vegetable oil, hydrogenated vegetable oil, lard, when fed for 3 days were equally effective in suppressing fatty acid synthesis from acetate by slices of rat liver. Our initial observations with mice,[5] however, suggested that at least in these animals different fats in the diet varied considerably in their ability to suppress fatty acid synthesis by liver slices. In the studies presented here we examined this problem further. Thus, we included in the experimental diets fats and triglycerides of predominantly different fatty acid compositions, so that we could obtain information as to possible differences between the particular fatty acids in their ability to influence hepatic fatty acid synthesis.

Allman and Gibson (17) have reported that dietary addition of corn oil or methyl linoleate, but not of methyl palmitate or methyl oleate, lowered within 2 days the high level of fatty acid synthesis observed in livers of mice that had been made deficient in essential fatty acids (by feeding for 18 days a diet containing 2% coconut oil as its only source of fatty acids). We observed the effects of feeding various fats without prior inducement of a deficiency syndrome, and the effects of refeeding after fasting. Jansen et al. (7) have recently reported similar work, but they measured lipogenesis from glucose

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[4] Gould, R. G., and C. B. Taylor 1950 Effect of dietary cholesterol on hepatic cholesterol synthesis. Federation Proc., *9*: 179 (abstract).

[5] Unpublished observations.

in vivo, rather than from acetate in vitro as we have done. Our results were essentially similar to theirs.

EXPERIMENTAL

Animals and diets. Male mice of the C57 L/J strain were obtained at weaning.[6] They were housed at constant temperature and illumination in individual cages, with sawdust bedding, and when not receiving an experimental diet they were offered a commercial laboratory ration[7] ad libitum. Water was freely available at all times.

The experiments were divided into two parts. In experimental series 1 we examined the inhibition of hepatic fatty acid synthesis from acetate caused by feeding for 1 to 5 days a high fat diet (15% corn oil) as opposed to a fat-free diet, and the effects of these two diets (table 1) when offered after either 1 or 2 days of fasting. In experimental series 2 we examined the effects on fatty acid synthesis of including in the diet, for 3 days, the following substances: corn oil,[8] safflower oil,[9] coconut oil,[10] tricaprylin, tripalmitin, triolein, oleic acid and mineral oil. These were all included as 10% of the diet, replacing an equal weight of cellulose from the fat-free diet shown in table 1. All experimental diets were offered ad libitum, and we measured the amount eaten by each animal.

In all cases the mice were killed between 8:30 and 10:00 AM, and for those fasting all food, but not water, was removed at the appropriate time prior to this.

TABLE 1
Diets

Ingredient	Composition	
	Fat free	High fat
	%	%
Glucose	50	50
Casein [1]	22	22
Salt mixture [2]	6	6
Defatted liver [3]	2	2
Vitamin B mixture [4]	1	1
Cellulose [5]	19	4
Corn oil [6]	0	15

[1] Vitamin-free casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Salt mixture USP XIV, Nutritional Biochemicals Corporation.
[3] Jayron powder, VioBin Corporation, Monticello, Ill.
[4] Hill, R., N. Baker and I. L. Chaikoff 1954 J. Biol. Chem., 209: 705 (see ref. 35).
[5] Alphacel, Nutritional Biochemicals Corporation.
[6] Mazola, Corn Products Company, New York, N. Y.

Incubation procedures. The animals were killed by cervical fracture, and their livers quickly removed and placed into ice-cold, Krebs-Henseleit, bicarbonate buffer (18), pH 7.3. Tissue slices, approximately 0.4 mm thick, were cut with a McIlwain-Buddle tissue chopper (19). For experimental series 1 we used 200 mg of slices, with 2.0 ml of the Krebs-Henseleit buffer, containing 4.0 μmoles of potassium 1-^{14}C-acetate. In experimental series 2 we used 100 mg of slices, 1.0 ml of buffer, and 2.0 μmoles of potassium 1-^{14}C-acetate. All incubations, in duplicate, were performed in glass, center-well flasks (20) fitted with self-sealing rubber serum caps, and were carried out under an atmosphere of 95% O_2 and 5% CO_2. The flasks were shaken mechanically for 2 hours at 37°. Metabolism was stopped by injection, through the rubber cap, of either 1.0 or 0.5 ml of 0.2 N H_2SO_4. A previous report (21) described in detail procedures for the extraction and counting of ^{14}C-labeled CO_2, long-chain fatty acids and cholesterol.

RESULTS AND DISCUSSION

Effects of dietary fat (15% corn oil), with and without prior fasting. Figure 1 shows the fluctuations in body weights and relative liver weights of mice that were fed the high fat and fat-free diets (table 1) for up to 5 days, following zero, 1 and 2 days of fasting. These experiments involved 64 mice. Just as Jansen et al. (7) had found, the animals receiving the high fat diet put on slightly more weight, and recovered weight more quickly after fasting, than did those receiving the fat-free diet (fig. 1). These differences, though not statistically significant, probably reflect the higher caloric value of the high fat diet, since there were no appreciable differences between the amounts eaten of the two diets.

The conversion of 1-^{14}C-acetate to CO_2, long-chain fatty acids and cholesterol by

[6] Jackson Laboratory, Bar Harbor, Me.
[7] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.
[8] Mazola, Corn Products Company, New York, N. Y.
[9] Pacific Vegetable Oil Corporation, Richmond, Calif.
[10] Durkee Famous Foods, Glidden Company, Berkeley, Calif.

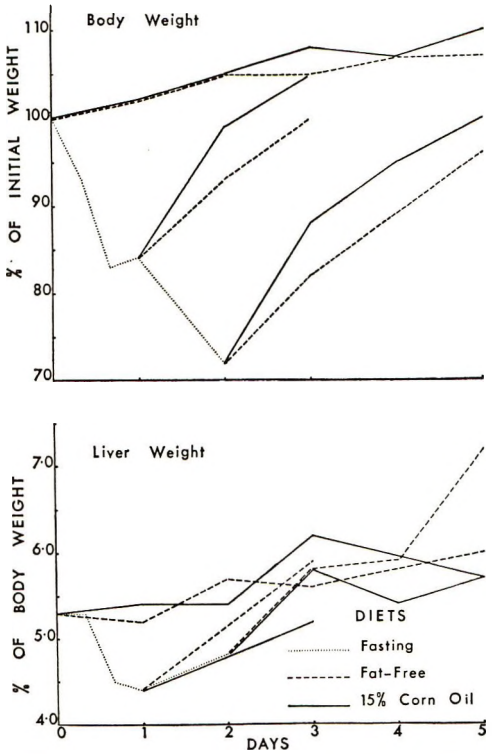

Fig. 1 Effects of fasting and of dietary fat on liver and body weights of mice. C57 L/J mice were fed either a fat-free or a high fat (15% corn oil) diet, with and without prior fasting. Body weights were calculated as a percentage of starting weight of each animal, liver weights as a percentage of body weight at killing. Each point represents the mean of all determinations. The study involved 64 animals.

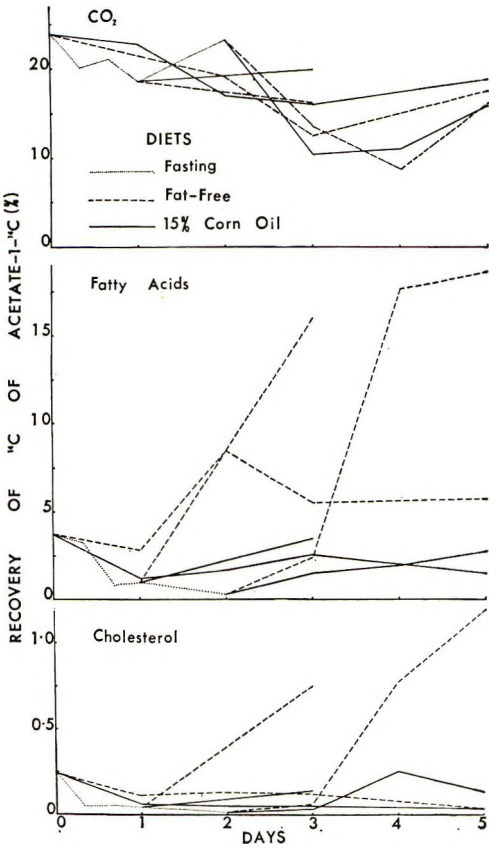

Fig. 2 Effects of fasting and of dietary fat on conversion of 1-^{14}C-acetate to CO_2, long-chain fatty acids and cholesterol by slices of mouse liver. C57 L/J mice were fed either a fat-free or a high fat (15% corn oil) diet, with and without prior fasting. Liver slices were incubated, in duplicate, with 1-^{14}C-acetate, as described in text. Each point represents the mean of determinations on from 2 to 5 animals, with a total of 64 animals involved. Application of the t test revealed the following statistical significances for differences in levels of fatty acid synthesis between livers of animals on different diets: a) high fat versus fat free, at all time intervals, 5% or better; b) stock ration versus 16-, 24- and 48-hour fasting, 2% or better; c) stock ration versus fat free, not significant; d) stock ration versus high fat, 5% or better, except day 3; e) fasting versus fat free, 1% or better; and f) fasting (48 but not 24 hours) versus high fat, 2% or better.

liver slices from these animals is shown in figure 2. As was expected (4, 7, 8, 22, 23) fasting caused a pronounced fall in hepatic synthesis of fatty acids.[11] Refeeding with the fat-free diet brought about a great increase in synthesis, to a level well above that of the control value, i.e., the value found for animals on a stock ration. This increase is the "adaptive hyperlipogenesis" described by Tepperman and Tepperman (3). Recovery of fatty acid synthesis, following 1 day of refeeding the fat-free diet after 2 days of fasting, was to about the same level as that found after a fast of 1 day. As seen in figure 1, the recovery of body weight on refeeding after fasting showed this same pattern.

In contrast to the fat-free diet, the high fat diet caused substantially lower levels of fatty acid synthesis, when offered both with and without prior fasting (fig. 2). Thus, the corn oil both caused a depression in synthesis from the control (stock ration)

[11] In this paper "synthesis" of fatty acids or cholesterol refers to the incorporation by tissue slices of the ^{14}C of 1-^{14}C-acetate into fatty acids and cholesterol, respectively.

level, and very greatly reduced the increase from the fasting level found with animals refed the fat-free diet. The experiments of Jansen et al. (7), when corn oil was fed for 3 days, showed the same results. In our experiments the difference between the effects of the fat-free and the high fat diets was clearly evident even after 1 day, and was more pronounced after 2 or more days. We have already reported (21) that the depression of fatty acid synthesis caused by feeding this high fat diet can be reversed within 3 days by feeding the fat-free diet.

In general, the changes in cholesterol synthesis (fig. 2) paralleled the changes in fatty acid synthesis—a fall on fasting, a great increase on refeeding with the fat-free diet and a much smaller increase with the high fat diet. In these experiments, and in those of Jansen et al. (7) with mice, the high fat diet suppressed cholesterol synthesis, whereas with rats Hill et al. (10, 15), using similar diets, found an increase in hepatic synthesis of cholesterol from acetate after feeding fat. We have no explanation of this, apart from a possible species difference. Many other workers have looked for a relationship between the rates of synthesis of fatty acids and of cholesterol in both rats and mice, but with conflicting results. Bortz (24) has recently discussed this work.

The changes in acetate oxidation (fig. 2) caused by the various dietary regimes may reflect some alteration in the overall operation of the acetate-activating and acetate-oxidizing enzymes of these livers. These alterations, however, do not appear to be of sufficient magnitude, nor consistently in the same direction, to account for the differences in lipogenesis.

Effects of various vegetable oils, triglycerides, oleic acid and mineral oil. Diets containing these substances (at 10%) were offered for 3 days, without prior fasting. Synthesis of fatty acids from acetate by liver slices was compared between these animals and those receiving the fat-free diet. Table 2 shows that there were no significant differences between the intakes of the various diets, nor between the liver weights or body weights of the animals eating these diets. Also, the ability of the liver slices to oxidize acetate was not appreciably altered by the various diets (table 2), indicating little or no significant changes in the operation of the acetate-activating and acetate-oxidizing enzymes.

By comparison with the fat-free diet, the only diets to cause significant depression of fatty acid synthesis from acetate by the livers of these animals were those containing corn oil or safflower oil (table 2). The other substances tested, coconut oil, tricaprylin, tripalmitin, triolein, oleic acid and mineral oil, all failed to suppress fatty acid synthesis.

The major proportions of the various long-chain fatty acids found in the dietary fats and oils used in these experiments are shown in table 3. It would appear that linoleic acid ($C_{18:2}$) in the diet may be of major importance in the "feedback" inhibition of hepatic synthesis of fatty acids, since only those diets high in this fatty acid were effective in reducing fatty acid synthesis. This is in agreement with the work of Allman and Gibson (17) and Allman et al. (25) with rats and mice, but not with that of Tepperman and Tepperman (26), Bottino et al. (27), Reiser et al. (28) nor Bhattathiry (29), all of whom used rats. The Teppermans (26) reported that in experiments similar to ours, but with rats, saturated coconut oil was equally as effective as, or even better than, corn oil in reducing the incorporation of ^{14}C-acetate into long-chain fatty acids by liver slices, although the reduction in lipogenesis was to about 50% of control values, compared with 13 to 22% in our experiments. Bottino et al. (27) found that with adult rats the inclusion in the diet of 15% tripalmitin, triolein or trilinolein all greatly depressed the incorporation in vivo of ^{3}H-acetate into carcass lipids. These workers, however, had fed the diets for 1 to 3 months, following a fat-free diet. Reiser et al. (28) found, also with rats, that triglycerides containing short-, medium- and long-chain fatty acids all depressed apparent hepatic fatty acid synthesis in vivo from ^{14}C-acetate, although trilinolein and safflower oil were the most effective. These diets, however, contained

TABLE 2

Effects of various dietary fats upon food consumption by C57 L/J mice, and on conversion of acetate-1-^{14}C to CO_2 and to long-chain fatty acids by liver slices from these animals

Dietary fat [1]	No. of animals	Body wt	Liver wt	Food consumed	Conversion of 1-^{14}C acetate to: [3]		P [4]
					CO_2	Fatty acids	
		g	*g*	*g/3 days*	%	%	
None	16	21.8 ± 0.6 [2]	1.3	12.2 ± 0.4 [2]	15.1 ± 1.2 [2]	6.0 ± 0.8 [2]	—
Corn oil	8	20.1 ± 0.6	1.1	12.0 ± 1.4	16.2 ± 1.7	0.8 ± 0.2	< 0.001
Safflower oil	11	20.6 ± 1.0	1.2	12.0 ± 1.0	16.7 ± 2.1	1.9 ± 0.4	< 0.001
Coconut oil	5	20.8 ± 0.4	1.3	12.9 ± 1.4	16.7 ± 2.2	4.5 ± 1.0	ns
Tricaprylin	10	20.2 ± 0.6	1.2	11.7 ± 0.6	17.1 ± 1.3	3.5 ± 0.5	ns
Tripalmitin	10	21.6 ± 0.8	1.3	14.1 ± 0.5	15.0 ± 2.2	5.6 ± 1.9	ns
Triolein	8	20.1 ± 0.5	1.3	11.3 ± 1.0	18.1 ± 1.5	4.4 ± 0.6	ns
Oleic acid	5	19.8 ± 0.6	1.5	11.8 ± 1.4	22.4 ± 2.9	8.7 ± 1.0	ns
Mineral oil	6	20.8 ± 0.4	1.3	12.6 ± 0.7	17.6 ± 2.4	5.4 ± 0.8	ns

[1] Various fats (at 10% of diet) were fed for 3 days.
[2] Values given are averages ± SEM.
[3] Liver slices were incubated in duplicate, with 1-^{14}C-acetate as described in text.
[4] P values are significances of differences for fatty acid synthesis between each diet and the fat-free diet; ns = not significant. Results were subjected to an analysis of variance following arc-sine transformation.

TABLE 3

Fatty acid composition of dietary oils

Fatty acid [1]	Percentage of total fatty acids		
	Coconut oil [2]	Corn oil [3]	Safflower oil [4]
$C_{8:0}$	5.2		
$C_{10:0}$	5.6		
$C_{12:0}$	43.8		
$C_{14:0}$	20.8		0.5
$C_{16:0}$	11.1	12.6	17.4
$C_{16:1}$		0.1	
$C_{18:0}$	10.0	2.3	1.8
$C_{18:1}$	3.5	28.5	10.0
$C_{18:2}$		54.0	67.9
$C_{18:3}$		2.5	2.4
$C_{20:4}$		0.1	

[1] Number of carbon atoms:number of double bonds.
[2] Analysis by courtesy of Dr. M. A. Williams, Department of Nutritional Sciences, University of California, Berkeley.
[3] Determined by the authors, using gas-liquid chromatography following saponification (30% KOH at 90°) and formation of the methyl esters as previously described (36).
[4] Castor, W. O., H. Mohrhauer and R. T. Holman 1966 J. Nutr., *89*: 217 (see ref. 37).

30% fat, and were fed for 2 weeks. Bhattathiry (29) found that at a dietary level of 10% both palm oil and olive oil (neither of which contains appreciable linoleic acid) depressed hepatic synthesis of fatty acids in vitro in weanling and adult rats, but not to the same extent as we are reporting here for diets containing linoleic acid.

It has become increasingly clear from all this work that any alteration in hepatic lipogenesis subsequent to the inclusion of fat in the diet will depend not only on the fatty acid composition of the fat, but also on the level in the diet, the time for which it is fed, the animal species and age examined, and whether the studies are conducted in vivo or in vitro.

Several years ago Bortz et al. (16) reported that the block in fatty acid synthesis caused by feeding fat was localized at the enzyme, acetyl-coenzyme A carboxylase (EC. 6. 4. 1. 2). Since that time many workers (30–32) have shown that this enzyme can be inhibited in vitro by adding long-chain fatty acids or fatty acyl-coA derivatives to the incubation medium. Recently Bortz (24) has shown an increase in the hepatic level of acyl-coA within 2 hours of feeding fat (corn oil), with a concomitant reduction in fatty acid synthesis. This is strong evidence

that the "feedback" inhibition of synthesis of fatty acids caused by feeding fat is due to an increased tissue level of acyl-coA, which in turn inhibits acetyl-coA carboxylase. Taketa and Pogell (33) and Dorsey and Porter (34), however, have recently argued strongly against ascribing a regulatory role in vivo to acyl-coA on the basis of its inhibitory effect on a wide range of enzyme systems.

There appears to be no ready explanation as to why, in our experiments, the various dietary fats differed in their ability to suppress fatty acid synthesis. As discussed above, there is some evidence that linoleic acid may be important, or even essential, but this evidence is not conclusive. Because we did not measure the fatty acid composition of the livers, we are unable to say what effect the various fats and oils in the diet had on the relative proportions of the various fatty acids present in the tissue. The different diets could have differentially affected linoleate utilization or metabolism. It is also possible that the intestinal absorption of the fats varies, the "nonsuppressors" being absorbed less. This would most certainly be the case with the mineral oil. We have no evidence for or against this point except to note that all diets were equally effective in maintaining body weight (table 2), so differential absorption, if present, may have been of only limited extent. Bortz and Lynen (30) have shown that different acyl-coA derivatives vary in their ability to inhibit acetyl-coA carboxylase, with the longer-chain ones generally being more effective. Different dietary fats possibly may vary in their ability to give rise to those acyl-coA derivatives that are most effective in suppressing fatty acid synthesis.

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DDT Effect on Rats Raised on Alpha-protein Rations: Growth and storage of liver vitamin A[1, 2]

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ABSTRACT Rats have been raised on rations containing alpha-protein as the sole source of nitrogen, and growth and liver vitamin A levels have been observed as a function of the rate of methionine supplement in the presence and absence of DDT. Increasing the level of methionine supplement in increments from zero to 4 g/kg produced a progressive increase in the growth of male rats. Females showed maximum growth when the methionine level was 1.0 g/kg. In the absence of added methionine DDT depressed growth, but at levels greater than 1.0 g/kg DDT stimulated growth. The level of vitamin A stored in the liver was depressed by feeding DDT, with the response being dependent on the level of methionine in the diet. The most pronounced reduction was observed with the unsupplemented ration, and the DDT effect was virtually eliminated when methionine was added at a level of 4 g/kg. A mechanism has been proposed for this interaction based on the action of DDT and methionine on the processes responsible for the absorption and transport of vitamin A.

Certain pesticides have been used so extensively that they have become almost ubiquitous in the environment. Numerous allegations have been made as to the possible consequences of general exposure to these substances; however, little evidence is available to substantiate or refute these statements. This impasse stems from the fact that it is difficult to evaluate the toxicological hazard of a long-term, low-level exposure to a chemical, taking all possible variables into account.

One variable which, to date, has received little attention in this regard is nutritional status and its possible interaction with a toxic stress. Phillips (1) has demonstrated that DDT can reduce the extent to which dietary vitamin A is stored in liver. Dieldrin produced a comparable effect when fed in a low protein diet (2). Although the chemical levels used in these diets were high relative to those observed in foodstuffs, such observations illustrate the potential effect of a chemical on nutritional status. Conversely, it has been observed that the vitamin A status of an animal can influence the toxicity of bromobenzene (3). Thus, a toxic stress can influence nutritional status and, conversely, nutritional status can affect the response to a chemical.

In this laboratory we have chosen to explore the possible interaction between the nutritional stress of feeding poor quality protein, α-protein of soy beans, and the toxicological stress of DDT exposure. This particular protein is deficient in the sulfur-containing amino acids, especially methionine. Rats were raised on α-protein rations with varying levels of methionine supplements and the effect of DDT on growth and liver vitamin A levels noted.

EXPERIMENTAL

Ration composition. The semipurified ration described in a previous publication (4) was composed of 22% protein as α-protein,[3] 4% salts, 5% fat and 67% carbohydrate. Adequate amounts of micronutrients were added as pure compounds (4). Vitamin A was added as vitamin A acetate to give a level of 3000 μg/kg of ration. Analytical standard *p,p'*-DDT [4] was incorporated into the ration, dissolved in the oil to give a level of 150 ppm. Supple-

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[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] ESA Pesticide Reference Standard, City Chemical Corporation, New York, N. Y.

ments of DL-methionine were added at the expense of the protein.

Experimental design. This paper summarizes the results of two experiments varying only in the levels of methionine supplementation. In experiment 1 zero, 1.0, 2.0, and 4.0 g/kg were used; in experiment 2 zero, 0.5, 1.0, and 4.0 g/kg were used. Results of experiment 1 suggested that a supplement of 1.0 g/kg produced a significant response and hence the minimum level of methionine supplement was reduced in experiment 2. In both experiments these four ration levels were fed in the presence and absence of DDT, making a total of eight dietary groups in each study.

Eight (4 male and 4 female) 28-day-old weanling rats from our closed colony of Wistar rats were weaned to each of the experimental diets making a total of 64 rats in each experiment. Rations were fed ad libitum. The animals were housed in individual cages, weighed weekly and observed for the incidence of any gross symptoms of toxicity or nutritional stress. Food intakes were recorded in experiment 2. After 12 weeks the animals were killed and samples of liver tissue taken for analysis.

Analytical procedures. Vitamin A concentrations in liver were determined using a spectrophotometric procedure with corrections for irrelevant absorption (5). Absorption curves were determined with a spectrophotometer.[5] The procedures used for statistical analysis have been described by Li (6).

RESULTS

Growth. Growth curves illustrating the influence of the methionine variable are given in figure 1. Lower levels of methionine resulted in a longer lag phase. Because the growth rate in all dietary groups was essentially linear from 2 to 6 weeks after being placed on experiment, the weight gain over this period has been used to analyze the effects of the experimental variables.

The average weight gains for this time interval are listed in table 1. Statistical analysis has been conducted using observations from those dietary groups common to both experiments. The level of methionine supplement was found to have a highly significant effect on growth rate, which observation is consistent with that of Barnes et al. (7) that this amino acid is limiting in soybean protein. In addition, both sex and DDT were found to interact in a significant manner with the methionine variable (table 2).

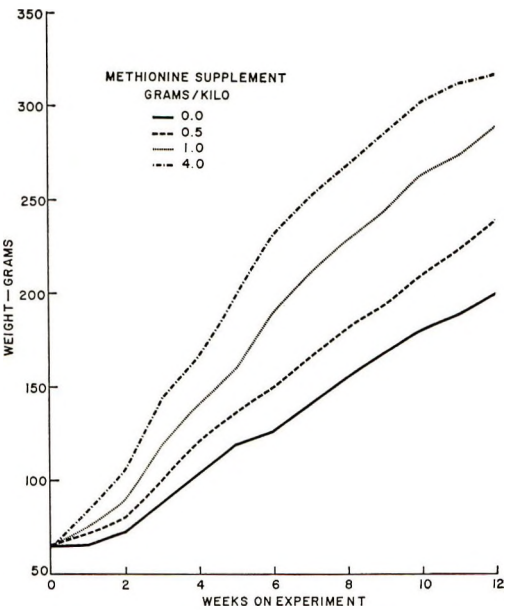

Fig. 1 Influence of methionine supplements on the growth of male rats raised on rations containing 22% α-protein and 150 ppm DDT.

Male and female rats responded quite distinctly to increasing levels of methionine supplement. Males showed a progressive increase in growth rate with the increasing levels of methionine supplement, whereas female rats showed a maximum growth rate when the methionine was added at the rate of 1.0 g/kg. The DDT interaction stems from the fact that in the absence of a methionine supplement a depression in growth rate is observed, whereas a stimulation of growth is observed in the presence of higher levels of a methionine supplement. A stimulation of growth has been observed in other studies in this laboratory using comparable levels of DDT. It is of interest to note that 0.5 g methionine/kg of ration produced an increase in growth rate, but was not sufficient to overcome completely the deleterious effect of the DDT (table 1, exp. 2).

[5] Cary Model 11, Cary Instruments, Monrovia, Calif.

TABLE 1

Effect of methionine supplement and DDT on growth — average weight gains (g) from 2 to 6 weeks

Sex	DDT level	Methionine level (exp. 1)				Methionine level (exp.2)			
		0	1.0	2.0	4.0	0	0.5	1.0	4.0
	ppm	*g/kg*	*g/kg*	*g/kg*	*g/kg*	*g/kg*	*g/kg*	*g/kg*	*g/kg*
Male	0	63	90	110	120	55	81	91	116
	150	45	97	121	135	53	70	103	126
Female	0	62	70	61	56	61	65	74	73
	150	52	82	77	70	50	59	76	70

TABLE 2

Methionine and sex, and methionine and DDT — interactions influencing growth rate — mean weight gain from 2 to 6 weeks

Methionine level	Sex		DDT level	
	Male	Female	0	150
g/kg			*ppm*	*ppm*
0	53	56	60	49
1.0	95	75	81	89
4.0	124	67	91	100

Least significant difference, 8 g ($P = 0.05$); and 11 g ($P = 0.01$).

TABLE 3

DDT and methionine effect on average daily food intakes (g solids/day)

DDT level	Methionine level			
	0	0.5	1.0	4.0
ppm	*g/kg*	*g/kg*	*g/kg*	*g/kg*
0	14.7	14.9	15.3	15.5
150	13.8	14.9	15.3	15.3

Least significant difference, 1.2 g ($P = 0.05$).

Only the methionine variable and, of course, sex were found to have a significant effect on the average daily food intakes calculated over the 12-week feeding period (table 3). Data from males and females are combined and differences due to DDT and the methionine variable are listed. There is a suggestion that DDT reduced food intake in the absence of the methionine supplement, but the difference observed was not statistically significant.

Liver vitamin A storage. The data listed in table 4 summarize mean values for total liver vitamin A as influenced by the level of methionine supplement and by the level of DDT. The data from each experiment were analyzed statistically and, in addition, an analysis was made using observations from those dietary groups common to both experiments. Sex produced no significant effect when "total liver vitamin A" rather than "mass per unit of tissue" was used as an index of storage level; hence, this variable is not incorporated in table 4, the values for males and females being combined. Comparable relationships are observed when vitamin A levels are expressed as mass per liver per 100 g body weight.

From the results of experiment 1 it is apparent that DDT reduced the level of liver vitamin A and that methionine added at the rate of 1.0 g/kg alleviated this condition somewhat. In the absence of DDT

TABLE 4

Influence of methionine and DDT on total liver vitamin A (mg/liver)

Methionine level	DDT level (exp. 1)		DDT level (exp. 2)		DDT level (composite)	
	0	150	0	150	0	150
g/kg	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
0	0.590	0.163	0.591	0.282	0.591	0.222
0.5	—	—	0.723	0.318	—	—
1.0	0.664	0.416	0.679	0.414	0.674	0.414
2.0	0.614	0.414	—	—	—	—
4.0	0.614	0.607	0.907	0.838	0.763	0.723
Least significant difference,						
$P = 0.05$	0.118		0.183		0.125	
$P = 0.01$	0.158		0.244		0.166	

the level of vitamin A in the liver was independent of the rate of methionine supplement. The data from experiment 2 were somewhat more variable and a significant effect of methionine on liver vitamin A was observed in the absence of DDT. In comparison with experiment 1 a supplement of 4.0 g methionine/kg produced rather higher levels of liver vitamin A, other values being fairly comparable. In the presence of DDT, methionine had to be added at a level greater than 1.0 g/kg to produce a significant increase in liver vitamin A over that observed in the unsupplemented group of animals. It was most significant, however, that in both experiments, DDT produced a marked reduction in the level of liver vitamin A, except when methionine was added at a level of 4.0 g/kg.

The analysis of composite data from both experiments indicated that in the absence of DDT, increasing the level of methionine supplement from zero to 4.0 g/kg produced a 30% increase in the level of vitamin A in the liver. Koyanagi and Odagiri (8) have reported a similar response. In the presence of 150 ppm DDT the same change in dietary methionine produced a threefold increase in the level of liver vitamin A. DDT produced a marked reduction in vitamin A storage except for the dietary group receiving the highest rate of methionine supplement. With the possible exception of the unsupplemented groups, food intakes cannot be a factor in interpreting these results (table 3). These data would both confirm the observations of Phillips (1) and at the same time introduce another factor — the methionine status of the animal — upon which the DDT response depends.

DISCUSSION

The fact that DDT depressed growth on an α-protein ration would suggest that DDT reduces the biological value of this protein, possibly by imposing some additional demand for methionine, or interfering with its utilization. In view of the complex nature of amino acid interactions and definition of protein values, such a conclusion would be somewhat premature and more intensive studies would be required before any definitive statements could be made concerning this interaction. The situation is complicated further by the fact that with higher levels of methionine in the diet DDT can stimulate growth.

To account for the DDT-methionine interaction as it influences vitamin A storage, one might postulate that methionine is required both for the detoxication of DDT and the storage of vitamin A. The additional demand imposed by the toxic stress could then limit the storage of vitamin A. This hypothesis, however, would not be consistent with our observation that an increase in the rate of methionine supplement also increased the amount of DDT and its metabolites stored in the liver (9).

An alternative and more plausible hypothesis is that both DDT and methionine interact in some fashion in the absorption and transport of vitamin A. This process, recently reviewed by Roels (10) involves first the hydrolysis of the ester to the alcohol, in which form it is absorbed into the mucosal cell and esterified with a fatty acid, predominantly palmitic acid. It is stored in the liver in this form after transport via the lymphatics and the blood stream. The transfer of the vitamin A ester from the mucosal cell into the lymphatics is dependent on chylomicron formation (11) and appears to be the limiting step in the absorption and transport of vitamin A (12). Chylomicron formation and subsequent lipid transport depend on the synthesis of the required proteins in the intestine (13), and ethionine — a methionine antagonist — inhibits lipid transport through this mechanism (14). Thus, limiting levels of methionine would be expected to influence the formation of chylomicrons, and consequently, the transport of the vitamin A ester. Further experiments would be required to establish if amino acids other than methionine would produce a comparable response.

At present it is not clear whether the vitamin A ester is carried in the chylomicron in simple lipid solution or bound to some specific acceptor protein. The formation of such a complex may be necessary to protect the compound from breakdown as has been postulated for vitamin A alcohol which is bound to a specific acceptor protein in blood plasma (15). Ganguly et al. (16) have suggested that the

level of specificity observed in the absorption of carotenoids and vitamin A might be explained by the formation of such complexes. Such a protein might be synthesized in the intestine and could also be dependent on the availability of methionine. DDT could affect the association of the vitamin A ester with the chylomicron or its binding to some acceptor protein.

The ability of some chemical to influence vitamin A storage through such a mechanism would depend on its affinity for and effect on the acceptor system, with a response being observed only under conditions where the transporting system became limiting. Such a situation may be achieved either by the administration of high levels of vitamin A, or by reducing in some fashion the level of the acceptor. The former condition is illustrated by some of Phillips' data (1) taken from rats which had been raised for 60 days on rations which contained 100 ppm of DDT (table 5). DDT produced a significant depression in liver vitamin A when a single oral dose of the vitamin was given and the rats killed 24 hours later. Under these conditions the DDT response was more pronounced with the higher dosage of vitamin A. DDT had no effect on the storage of vitamin A when the two were fed concomitantly in the diet. In this instance the rats would be ingesting in small increments about 27 to 30 μg vitamin A a day, assuming a daily intake of 15 g of solids. With such a low rate of intake one could postulate that the level of acceptor system was not limiting even in the presence of the DDT and no effect on storage results. The reason that DDT produced an effect in our studies could be attributed to a limiting level of the acceptor system resulting from the protein stress. In addition the dietary level of vitamin A was somewhat higher, 3000 against 1800 μg/kg.

TABLE 5

Influence of rate of vitamin A intake on DDT effect [1]

DDT level	Total liver vitamin A, μg		
	Single dose		Concomitant feeding
	260	190	27
ppm	*μg*	*μg*	*μg/day (est.)*
0	35.4	11.0	169
100	11.4	6.7	165
Percentage reduction	68	39	0

[1] Phillips, W. E. J. 1963 Can. J. Biochem. Physiol., *41:* 1793.

Compounds other than DDT should be active if the interaction is not stereospecific. Thus, it is of interest to note that dieldrin also reduced the level of liver vitamin A, and that the response was more pronounced when the dieldrin was fed in a low protein diet (2).

Although other possibilities exist, the proposed mechanism can account for present experimental data. Kinetic studies in rats with lymph duct fistulas are needed to confirm the basic hypothesis. Also, information is required on the nature of the association of vitamin A ester with chylomicrons, and how DDT might affect such a system.

One must reserve judgment on the public health implication of this interaction pending the completion of further experiments designed to define the minimum effect levels of DDT as they are influenced by protein nutrition.

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Effect of Thiamine Deficiency in Rats on Adipose Tissue Lactate Dehydrogenase Isozyme Distribution [1]

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ABSTRACT Lactate dehydrogenase (LDH) (EC. 1.1.1.27) was present in five isozymic forms in rat epididymal adipose tissue from rats fed a complete diet; LDH-I (most anodal electrophoretically) was present in low concentration. Thiamine deficiency resulted in a reduction of specific activity (units per milligram soluble protein) of total LDH, but a marked increase in LDH-I and LDH-II. Feeding thiamine-deficient rats a complete diet restored the LDH-isozyme distribution pattern to control characteristics. The shift in LDH-isozyme distribution pattern with thiamine deficiency was suggestive of an increase in the activity of the gene responsible for the synthesis of the H subunit.

Recent work from this laboratory (1) demonstrated that lactate dehydrogenase (LDH) (EC. 1.1.1.27) was present in five isozymic forms in rat epididymal adipose tissue, with LDH-I (most anodal electrophoretically) present in lowest concentration in normal rats. The relative distribution of the five LDH isozymes was significantly altered by metabolic stress such as prolonged fasting (1) or diabetes.[2] Such variation in relative concentration of the LDH isozymes implies some type of selective control of enzyme synthesis. Since each isozyme is a tetramer composed of varying amounts of two polypeptide subunits (H and M), one means of control of the concentration of the LDH isozymes would be through a regulation of the two genes which are responsible for the synthesis of the two polypeptide subunits (2). The distribution pattern occurring during metabolic stress was shown to be due to an increase in the H subunit (1). Since LDH catalyzes the reversible reaction between pyruvate and lactate, it was considered that the increase in pyruvate associated with thiamine deficiency might be involved (by induction) in a change in the isozyme distribution of this enzyme. Van Eys (3) earlier showed that total lactate dehydrogenase was lowered in tissues from thiamine-deficient rats. The present paper reports the effect of thiamine deficiency in rats on the relative concentration in adipose tissue of the five isozymes of lactate dehydrogenase.

EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain were maintained at a temperature of 21 to 22° and a relative humidity of 40 to 45%. They were fed a commercial thiamine-deficient diet [3] beginning at age 32 days at which time they weighted 80 to 90 g. Control animals were given a complete diet ad libitum. Animals allowed to recover dietarily from their thiamine-deficient condition were given the deficient diet to which had been added 44 mg thiamine/kg diet. The rats were killed by decapitation and exsanguination; they were always killed at 9 AM to avoid possible diurnal variation in enzyme levels (4). The epididymal fat pad was removed in its

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[2] Moore, R. O., and F. D. Yontz, unpublished observations.

[3] The thiamine-deficient diet was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The diet consisted of vitamin test casein, 18%; sucrose, 68%; vegetable oil, 10%; salt mixture no. 2, USP XIII, 4% (calcium biphosphate, 13.58%; calcium lactate·$5H_2O$, 32.70%; ferric citrate·$5H_2O$, 2.97%; magnesium sulfate, 13.70%; potassium phosphate, dibasic, 23.98%; sodium biphosphate·$2H_2O$, 8.72%; and sodium chloride, 4.35%); to each 100 lb. of the above was added: (in grams) vitamin A conc (200,000 U/g), 4.5; vitamin D conc (400,000 U/g), 0.25; alpha-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; and calcium pantothenate, 3.0; (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35.

entirety, freed of major blood vessels, weighed, and homogenized in a cooled Potter-Elvehjem homogenizer in 1 or 2 volumes of 0.1 M KH_2PO_4–NaOH buffer, pH 7.5. The homogenate was centrifuged at 27,000 × *g* for 30 minutes at 4°. The supernatant fraction was assayed for protein content (5) and total LDH activity, and used as source material for the electrophoretic separation of the LDH isozymes.

Horizontal cellulose acetate electrophoresis was used to determine the LDH isozyme distribution pattern. The electrophoretic separation was conducted for 140 minutes at 200 v in barbital–sodium barbital buffer, pH 8.6, ionic strength 0.075; the system was maintained at 8°. The LDH isozyme activity positions were visualized using nitroblue tetrazolium as a final electron acceptor (6) during an incubation of 30 minutes. The intensity of the activity bands was estimated using a recording electrophoresis densitometer, and quantitated by planimetry. Individual isozyme activity was expressed as a percentage of total (five isozymes) LDH activity.

Total LDH activity was measured by estimating the disappearance of DPNH at 340 mμ in a double-beam recording spectrophotometer. The reaction mixture contained the following in a final volume of 3 ml: 0.29 mmole KH_2PO_4–NaOH (pH 7.5), 16.5 × 10^{-2} μmoles sodium pyruvate, 0.39 μmole DPNH and 20 μl of the enzyme solution. The final concentration of the enzyme was such that the reaction was linear with time over the period measured. A unit of LDH activity was defined as the amount of enzyme which causes an initial rate of oxidation of 1 mμmole DPNH/minute under the conditions specified at 25°.

RESULTS

While on the thiamine-deficient diet, the rats in the experiments began to lose weight 14 days after the beginning of the diet. Because it was considered that sufficient epididymal adipose tissue for conduct of the desired assays was not obtainable from rats of 120 g or less body weight they were given a complete ration on day 14 for 1 day; thereafter, they again began losing weight about 24 days after the initial beginning of the diet (fig. 1). Using body weight as a gross indication of the state of deficiency, the first estimation of epididymal adipose tissue lactate dehydrogenase and its isozyme distribution pattern in "deficient" animals was made 27 days from the initial feeding of the thiamine-deficient diet. Thereafter, similar assays were performed on two deficient and the appropriate control rat at 2-, 3-, or 4-day intervals to the end of the experimental period (49 days from the initial feeding). The data were recorded for individual rats. Thirty-eight days after the initial feeding of the deficient diet, at which time the animals showed severe deficiency symptoms, some of the rats were given a complete diet ad libitum. These rats were also used for adipose tissue LDH assay at several-day intervals for a period of 21 days.

The specific activity of adipose tissue total LDH relative to tissue soluble protein was not significantly different from that of control tissue during the first 30 days on the thiamine-deficient diet. Thereafter, the specific activity of the total LDH was significantly lower ($P < 0.001$), and remained low throughout the remainder of the feeding period, although there was a return toward the control levels near the end of the feeding period (fig. 1). Thiamine-deficient rats which were returned to a complete diet recovered their adipose tissue total LDH in about 8 to 10 days, after which they were not significantly different from control rats in this regard.

The adipose tissue total LDH per unit of tissue weight increases during the period of thiamine deficiency and becomes highly significant ($P < 0.005$) after 35 days on the deficient diet. This expression of data relative to adipose tissue weight is of little metabolic significance, since it reflects largely a change in tissue weight due to fat mobilization.

The adipose tissue LDH isozyme distribution pattern for rats on a complete diet is one in which LDH-I (H_4) is present as only 5.4 ± 0.77% of the total LDH (table 1, fig. 2). In contrast, the adipose tissue LDH-I in rats that were fed a thiamine-deficient diet for 27 to 35 days was 12.7 ± 6.2% of the total LDH; in rats on the

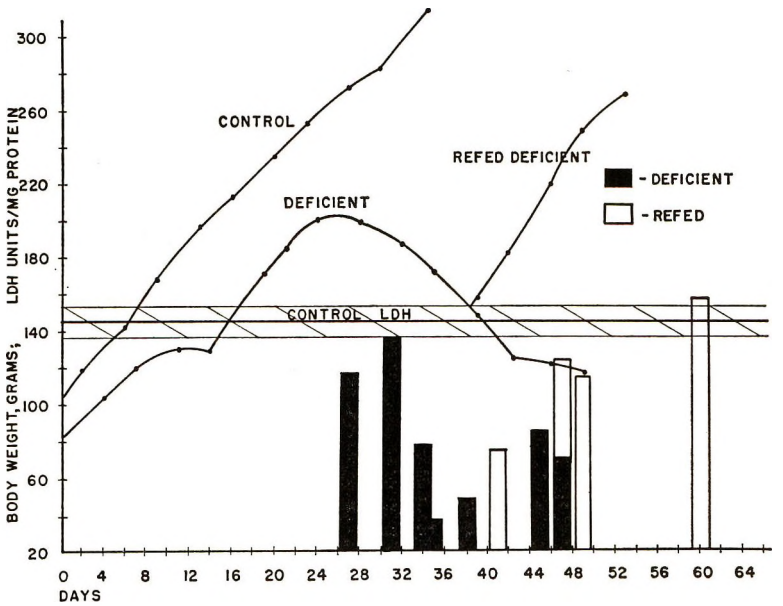

Fig. 1 Body weight and epididymal adipose tissue total LDH during the feeding of the thiamine-deficient diet. Method for assay of LDH is in the text. Horizontal shaded area is the mean ± standard deviation for the adipose tissue total LDH in control rats receiving a complete diet. Vertical columns are the mean of at least two rats (four assays) for adipose tissue total LDH for rats on a thiamine-deficient diet, or those "refed" a complete diet for the period indicated after having been on the thiamine-deficient diet for 38 days.

thiamine-deficient diet for 38 to 47 days this value was 24.6 ± 5.9% of the total LDH. Both of the thiamine-deficient values were significantly different ($P < 0.001$) from the control value.

Rats that were fed a complete diet after having been on the thiamine-deficient diet for 38 days returned toward the control values as the complete-diet feeding continued. Such rats, on the complete diet for 8 to 10 days had 9.8 ± 3.8% LDH-I in their adipose tissue; those on the complete diet 13 to 21 days had 8.2 ± 3.0% LDH-I. The latter value is not significantly different from the control value.

LDH-II, which is a tetramer composed of three H subunits and one M subunit (H_3M), was altered in adipose tissue of rats on a thiamine-deficient diet in a similar way to LDH-I, but to a lesser extent. LDH-II in adipose tissue from rats fed a complete diet (control) was 22.6 ± 5.3% of the total LDH; from rats fed a thiamine-deficient diet for 27 to 35 days 26.0 ± 5.5%; from rats fed a thiamine-deficient diet for 38 to 47 days 34.4 ± 3.5%. The latter value was very significantly different from the control ($P < 0.001$). Rats which were fed the complete diet after having been on the deficient diet for 38 days gradually returned to control concentrations of LDH-II. Such rats fed the complete diet for 13 to 21 days had 21.7 ± 3.8% LDH-II in their adipose tissue, a value nearly the same as that for control animals.

DISCUSSION

In the experiments reported, the observation was made that on a tissue weight basis the protein and total LDH activity of epididymal adipose tissue rose during the early period of thiamine deficiency indicating a retention of the components relative to fat. The specific activity of LDH (units per weight of soluble protein) was unchanged during this period, denoting that any movement of LDH from the tissue was at about the same rate as other protein. With more extended deficiency (greater than 30 days on the deficient diet) the lower specific activity of LDH might

TABLE 1

Distribution of LDH isozymes in rat adipose tissue and the effect of thiamine deficiency thereon

Days on deficient diet	Days on complete diet	No. of rats	Percent of LDH isozymes [1]				
			LDH-I	LDH-II	LDH-III	LDH-IV	LDH-V
0		18 [2] (control)	5.4 ± 0.77 [3]	22.6 ± 5.3	22.1 ± 9.0	25.6 ± 8.5	25.4 ± 4.8
27–35		12	12.7 ± 6.2	26.0 ± 5.5	18.5 ± 2.6	17.4 ± 5.5	24.9 ± 6.8
38–47		9	24.6 ± 5.9	34.4 ± 3.5	16.9 ± 5.0	10.5 ± 2.9	14.1 ± 5.0
38	2–4	4	14.9 ± 3.0	28.4 ± 4.6	23.6 ± 4.9	16.6 ± 5.4	16.6 ± 4.3
38	8–10	6	9.8 ± 3.8	22.8 ± 5.4	23.3 ± 5.9	19.6 ± 3.7	23.7 ± 7.2
38	13–21	6	8.2 ± 3.0	21.7 ± 3.8	16.8 ± 3.1	22.7 ± 7.2	29.7 ± 8.5

[1] Method for assay of LDH isozymes and description of diet is in the text.
[2] Control rats were fed the complete diet throughout the experimental period.
[3] Standard deviations follow the mean values.

Fig. 2 Percentage of total LDH as LDH-I in epididymal adipose tissue from thiamine-deficient rats. The method for assay of LDH-I and the feeding schedule are described in the text. Each column represents the mean value of at least two rats, four assays.

be interpreted to mean that LDH was leaving the cell more rapidly than other proteins, or that it was being synthesized at a lower rate. The return to control levels of the specific activity of total LDH, 8 to 10 days after deficient animals were allowed a complete diet, signifies a reestablishment of the normal protein and enzyme synthesis and degradation in the adipose tissue.

The assay (by electrophoresis) of the individual members of the LDH isozyme family allows more definitive information to be collected relative to alterations in adipose tissue LDH during a period of thiamine-free food intake. The increase in LDH-I provoked by thiamine deficiency suggests an increase in the activity of the gene responsible for coding of the H subunit relative to the gene coding for the M subunit. An increase in LDH-II (H_3M) might also be expected if the gene for the H subunit were more active. The increase in LDH-I during thiamine deficiency was significantly different from the control value prior to meaningful body weight loss, indicating that the change in LDH distribution was an early result of the thiamine deficiency. A shift in the distribution of LDH isozymes toward LDH-I would serve to limit the production of lactate in the tissue, since LDH-I was shown to be inhibited by pyruvate (7). Thus, normally in the presence of LDH-I the pyruvate

would be oxidized via the tricarboxylic acid cycle; however, the requirement for thiamine pyrophosphate of pyruvate dehydrogenase would reduce the amount of pyruvate being oxidized, and provide for its accumulation and the subsequent derangement of numerous metabolic reactions. Alternatively, it is possible that in the onset of thiamine deficiency the pyruvate accumulates early and serves as a type of inducing agent for the H subunit of LDH. This possibility is currently being explored.

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Composition of α Complete Purified Equine Diet [1]

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ABSTRACT A complete, highly palatable, readily pelleted purified equine ration was developed. The ration consists of 16% vitamin-free casein, 40% glucose, 25% cornstarch, 13% alpha-cellulose, 1% fat, 2.4% dicalcium phosphate, 1% NaCl, 0.8% K and the following trace elements and vitamins: (in ppm) Mg, 390; Zn, 20; Mn, 26; Fe, 10; Cu, 5; Co, 1.5; I, 3.6; inositol, 400; choline, 240; niacin, 45; *p*-aminobenzoic acid, 22; thiamine, 15; riboflavin, 4.5; Ca pantothenate, 4.5; pyridoxine, 3.5; folic acid, 2; and menadione, 0.37; (in IU/kg) vitamin A, 4400; vitamin D_2, 520; and vitamin E, 44. This ration was consumed by suckling- and weanling-age foals in excess of 25 g/kg body weight per day and resulted in 1 kg gain/day equivalent to nursing foals. Hematologic and other clinical parameters were maintained within normal ranges and the diet resulted in the passage of formed stools. The diet is believed adaptable to most equine nutrient requirement studies except selenium.

Studies to ease the paucity of equine nutrient requirement data are dependent, in part, upon the availability of an acceptable functional complete purified equine ration. Such a ration could also facilitate use of the horse as a laboratory animal model for comparative pathophysiologic studies and have application in the postoperative dietary management of certain equine surgical cases. A highly successful and acceptable synthetic equine ration could even replace standard rations for pleasure horses housed under circumstances which discourage the use of natural feedstuffs.

This report pertains to the composition of a highly palatable, readily pelleted, complete purified equine diet developed over a 3-year period which permits growth equal to that of foals nursing dams, results in passage of formed stools with minimal odor and produces normal hematologic and clinical parameters.

EXPERIMENTAL

Forty-two orphaned foals of lighthorse conformation obtained at 1 to 10 days of age were utilized in the development of this ration. The performance data for the diet herein reported were obtained from 16 foals fed the diet for 120 days. The foals were taught to drink a commercial milk replacer which was subsequently dispensed by an automatic foal feeder.[3] The pelleted purified ration was made available to the foal upon its arrival. Within 2 to 3 days the foals started eating the pellets and the foals could be completely converted to the pelleted ration as the sole nutrient source by 21 days of age without growth impairment. Ad libitum feeding was practiced; the ration intake was calculated daily and the foals were weighed three times weekly. Blood samples for hematologic and serum studies were obtained every 2 weeks. The foals were housed in individual, unbedded 3.66 m by 3.66 m stalls, with concrete floors and walls and iron pipe, bull-pen-type doors. Molded plastic corner-feed troughs and galvanized-iron water pails were used. Exercise, unfortunately, was limited to that obtained in the stalls and while being weighed.

The ration was mixed in a 500-kg capacity horizontal mixer and pelleted in a commercial pelleting machine [4] using a 0.474 cm by 5 cm die. Prior to incorporating with the more bulky components of the ration, the trace and minor elements were premixed, the vitamins were mixed with water and absorbed into a small portion of the cellulose, and the cottonseed oil was also absorbed into a small portion of the

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[3] Stowe, H. D. 1967 Automated orphan foal feeding. Proc. 13th Annual Convention of American Association Equine Practitioners, New Orleans, Louisiana.
[4] Master model CPM, California Pellet Mills Company, Folsom and 14th Street, San Francisco, Calif.

cellulose. Three liters of water were used to incorporate the vitamins for a 500-kg mix and no further addition of water or steam was necessary to accomplish pelleting. In the absence of commercial pellet-cooling equipment, the pellets were allowed to cool and dry in 5- to 7.6-cm layers on a cement floor and subsequently placed in containers.

RESULTS

The composition of the ration found to meet the criteria for an acceptable purified equine ration is presented in table 1. This ration was consumed at the rate of 25.7 ± 1.5 g/kg body weight per day with a concurrent water consumption of 72 ± 6.0 ml/kg body weight per day. Under the conditions of the experiment foals fed this ration gained 1.03 ± 0.09 kg/day and required 2.61 ± 0.10 units of the ration per unit of gain during the 120-day feeding period.

The calculated quantities of vitamins consumed daily per kilogram body weight, based upon a ration intake of 25 g/kg body weight per day, are presented in table 2 and are compared, when possible, with the National Research Council's recommendations (1). Each vitamin is supplied in excess of the indicated requirements.

The hematologic data associated with the use of the purified ration are compared in table 3 with values cited for some comparably aged, naturally fed horses.

Selected electrolyte and clinical data obtained from foals being fed the purified ration are compared in table 4 with some values given in the literature for horses maintained under normal feeding conditions.

DISCUSSION

The emphasis in the development of this ration has been placed upon its early acceptability by suckling-age foals, and upon foal performance equal to that expected of nursing foals which, according to Stowe (11), approximates 1 kg/day during the suckling period.

It is believed that this ration is applicable to many specific equine nutrient requirement studies with the exception of selenium. The exception is due to the inherent selenium content of the casein.

This ration has a calculated digestible protein content of 15%. A foal gaining 1 kg/day and requiring 2.61 kg feed/kg gain would therefore consume 0.391 kg digestible protein per kilogram gain. This compares very favorably with the 0.405 kg extrapolated by Mitchell (12) to be the protein requirement per kilogram foal growth.

Ideally, one ingredient should serve as the sole carbohydrate source in the ration. With the pelleting equipment available, however, a diet containing either glucose

TABLE 1

Composition of complete purified equine diet

	kg		*g*
Casein, vitamin free	16.00	Inositol	40.0
Glucose	40.00	Choline chloride	24.0
Cornstarch	25.00	Niacin	4.476
Cellulose [1]	13.00	*p*-Aminobenzoic acid	2.200
Cottonseed oil	1.00	Thiamine·HCl	1.544
Dicalcium phosphate	2.40	Riboflavin·HCl	0.455
Sodium chloride	1.00	Calcium pantothenate	0.455
Potassium carbonate	1.415	Pyridoxine	0.356
		Folic acid	0.207
	g	Menadione	0.037
MgO	65.0	Vitamin A [2]	1.760
$ZnSO_4 \cdot 7H_2O$	8.8	Vitamin D_2 [3]	0.110
$MnSO_4 \cdot H_2O$	8.0	Vitamin E [4]	20.000
$FeSO_4 \cdot 7H_2O$	4.5		
$CuSO_4 \cdot 5H_2O$	2.0	Total	100 kg
$CoCl_2 \cdot 6H_2O$	0.6		
KI	0.5		

[1] Solka Floc, Brown Company, Boston, Mass.
[2] Two hundred fifty international units per milligram.
[3] Five hundred international units per milligram.
[4] Two hundred twenty-two international units per gram.

TABLE 2

Calculated daily vitamin intake of foals fed the purified diet compared with National Research Council recommendations

Vitamin	Intake/kg body wt per day	
	Calculated	Recommended
	μg	*μg*
Thiamine	385	55
Niacin	1102	100
Riboflavin	118	44
Calcium pantothenate	135	40
Pyridoxine	88	
p-Aminobenzoic acid	500	
Folic acid	54	
Inositol	10×10^3	
Choline-chloride	6×10^3	
Biotin	93×10^{-6}	
Cyanocobalamin	6×10^{-7}	
Menadione	9	
	IU	
Vitamin A	110	33
Vitamin D_2	13	6.6
Vitamin E	1.1	

or cornstarch as the sole carbohydrate portion presented difficult pelleting problems. The diet containing 65% glucose caramelized and burned in the pelleting die. The ration containing 65% cornstarch compacted so tightly and created so much friction in the die that it was impossible to pellet. The ratio of glucose to cornstarch finally used in the ration was selected because it imparted pelletability characteristics to the overall mixture.

Levels of cellulose in the diet between 10 and 15% were tried at various periods during the development of this ration. Under the conditions of these investigations, an increase in the fiber content of the purified diet beyond 13% resulted in the loss of stool formation. It is believed significant that 2- to 3-week-old foals readily accepted the pelleted, 13% cellulose ration as their sole nutrient source without growth impairment. The use of alpha-cellulose levels approaching the 24% level used by Teeter et al.[5] in mature horses was not attempted.

The young foal acceptance of this dry pelleted diet is of major practical significance in orphan foal management; i.e., in a matter of days, almost any orphan foal can be converted from the frequent, tedious, usually prolonged milk feeding regimen to the free choice pelleted diet regimen while maintaining normal growth. Such a program could well be considered in lieu of a nurse mare in any equine nursery.

The 1% fat content of this ration was established because reasonable growth and no essential fatty acid deficiency symptoms had been observed in horses fed for 1 to 2 years a 33% Torula yeast ration whose sole fat content was furnished by the 3% fat content of the yeast.

Feed grade dicalcium phosphate was utilized as a palatable, inexpensive source of calcium and phosphorus in a ratio approximating 1.5:1. The calcium and phosphorus intakes per kilogram gain are calculated at 16.9 and 11.3 g, respectively. These values compare very favorably with the requirements for 90- to 185-kg foals

[5] Teeter, S. M., M. C. Stillions and W. E. Nelson 1967 Recent observations on the nutrition of mature horses. Proc. 13th Annual Convention of American Association Equine Practitioners, New Orleans, Louisiana.

TABLE 3

Comparison of equine hematologic parameters associated with purified and natural diets

Parameter	Purified diet	Natural diet	Ref
Hemoglobin, g/100 ml	14.8 ± 0.3 [1]	12.9	Todd et al. (2)
PCV, %	35.3 ± 1.1	39	Todd et al. (2)
RBC, $10^6/mm^3$	9.2 ± 1.2	12.8	Todd et al. (2)
WBC, $10^3/mm^3$	8.8 ± 0.1	13.6	Todd et al. (2)
Neutrophils			
Segmented, %	50.7 ± 5.2	49	Schalm (3)
Bands, %	2.3 ± 0.7	0.5	Schalm (3)
Lymphocytes, %	44.3 ± 8.3	48	Schalm (3)
Monocytes, %	< 1	2	Schalm (3)
Eosinophils, %	< 1	4	Schalm (3)
Basophils, %	< 1	0.5	Schalm (3)

[1] SEM.

TABLE 4

Comparison of equine clinical parameters associated with purified and natural diets

Serum component	Diets		Ref
	Purified	Natural	
Calcium, mg/100 ml	11.6 ± 1.2 [1]	9–13	Altman and Dittmer (4)
Phosphorus, mg/100 ml	5.63 ± 0.23	3.1–5.6	Altman and Dittmer (4)
Sodium, mEq/liter	133 ± 3.0	146–152	Altman and Dittmer (4)
Potassium, mEq/liter	4.42 ± 0.1	4.1–4.6	Bruning [2]
Magnesium, mEq/liter	2.06 ± 0.01	1.6–2.5	Albritton (5)
Copper, μg/ml	2.13 ± 0.09	1.18–3.56	Stowe (6)
Bicarbonate, mEq/liter	23.0 ± 0.7	23–29	Spector (7)
Chloride, mEq/liter	86.6 ± 2.7	98–106	Spector (7)
Total protein, g/100 ml	7.3 ± 0.1	6.6–8.3	Spector (7)
Cholesterol, mg/100 ml	90 ± 10	83–140	Kritchevsky (8)
Bilirubin, mg/100 ml	1.43 ± 0.1	0.2–6.2	Cornelius et al. (9)
Selenium, μg/100 ml	6.26 ± 0.47	3.3–8.67	Stowe (10)
Iron, μg/ml	2.26 ± 0.2	1.78–2.96	Stowe [3]
Alkaline phosphatase, KAU/100 ml	32.5 ± 1.0	20–48	Stowe [3]

[1] SEM.
[2] Bruning, P. 1950 Potassium content of blood serum and cerebrospinal fluid in horses. Inaugural dissertation, University of Hanover, Germany.
[3] Unpublished data.

listed by the National Research Council (1).

Studies were not conducted relative to the optimal level of sodium chloride in this foal ration and perhaps the acceptability and performance of the diet could be maintained with less than 1% NaCl. However, NaCl has a sparing action upon potassium and it is possible that the NaCl over and above the usual 0.5% level in purified diets may be so used.

The potassium content of this ration is calculated at 0.8% and in this particular combination of ingredients approaches the optimal level for maintenance of palatability, normal growth and clinical parameters.[6]

The trace mineral content of this ration provides the following of each element: (in ppm) Mg, 390; Zn, 20; Mn, 26; Fe, 10; Cu, 5; Co, 1.5; and I, 3.6. The mean intake of each of these elements per kilogram gain is calculated as follows: Mg, 1017; Zn, 52; Mn, 74; Fe, 26; Cu, 13; Co, 2.9; and I, 10 mg.

Pearson et al. (13) and Stowe (11) have reported impaired performance of synthetic equine rations containing less than 10% yeast. On the basis that the impairment was associated with B-vitamin adequacy, the B-vitamin content of this purified diet was calculated to be equivalent to that supplied by the yeast in a 10% Torula yeast diet. The relatively high level of inositol in the ration, therefore, is a reflection of the inherent high inositol content of Torula yeast and not necessarily an indication of a high inositol requirement for the horse.

The hematologic data, while within respectable ranges for most purposes, suggest that foals fed this synthetic ration have a hyperchromic erythrocytopenia. Appropriate adjustment in the ration components affecting hematopoiesis appears in order providing packed cell volume (PCV) and the number of erythrocytes per unit volume could be elevated while at least maintaining hemoglobin levels.

With the exception of sodium and chloride, the electrolyte and clinical data are within reported ranges for horses on natural diets. However, because the serum sodium value minus the corresponding bicarbonate and chloride values of foals fed the purified ration is 23 as compared with 21 for naturally fed horses, an attempt to elevate serum sodium and chloride appears unwarranted.

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Effect of Restricted Energy and Protein Intake on Atherosclerosis and Associated Physiological Factors in Cockerels [1,2]

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ABSTRACT Practical or semipurified diets, with and without added cholesterol, were fed to growing cockerels to determine the effect of dietary energy or protein restriction on systolic blood pressure, total serum cholesterol, total serum lipids, liver lipids and incidence of atherosclerosis in thoracic and abdominal aortas. The effect of dietary protein or energy restriction on systolic blood pressure was inconsistent. Variations in blood pressure appeared to be associated more with body weight than with restriction of protein or energy as such. Serum cholesterol, serum lipids and liver lipids were markedly increased when cockerels were fed diets containing cholesterol. Each was increased by protein restriction and decreased by energy restriction. Without added dietary cholesterol, serum cholesterol was not significantly affected by protein or energy restriction. Serum lipids were significantly increased, and liver lipids were slightly increased, when birds were fed protein-restricted diets without added cholesterol. Atherosclerosis was more prevalent when the diets contained cholesterol, and more severe in the abdominal than in the thoracic aorta, regardless of dietary treatment. Protein or energy restriction affected atherosclerosis, especially in the thoracic aorta, most when the diets contained cholesterol. Incidence of atherosclerosis in the thoracic aorta was reduced in energy-restricted, and increased in protein-restricted birds.

The effect of altered nutritional status on atherosclerotic involvement and serum cholesterol concentration in the chicken has been the subject of several investigations. One procedure used has been to restrict the total dietary intake to a certain amount (66, 70 or 80%) of that consumed by full-fed birds. This form of food restriction had no effect on serum cholesterol concentration unless cholesterol was included in the diet. In one study, however, abdominal aortas from food-restricted birds showed less atherosclerosis than those from full-fed birds (1).

Nutrient restriction also has been accomplished by withholding food for a given period, such as feeding birds every other day or once or twice each day (2–5). This form of restriction resulted in increased serum cholesterol levels and more severe aortic and coronary atherosclerosis when atherogenic diets were fed.

Other investigators have examined more specifically the effects of restricted protein intake (6–16). When atherogenic diets were fed, amino acid inadequacies and lowered dietary protein levels led to increased serum cholesterol concentrations and increased aortic and coronary atherosclerosis.

In studies reported, the effect of calorie intake on serum cholesterol concentration and atherosclerosis has usually been confounded with concomitant variations in dietary fat levels or restriction of other nutrients.

The present series of experiments was conducted to study the effects of dietary energy and protein restriction per se on atherosclerosis and associated factors in the adolescent cockerel.

EXPERIMENTAL PROCEDURE

Commercial broiler strain, male chickens were used in four experiments. One-day-old chicks were battery-reared and

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received adequate nutrition and management until they were put on experiment. During the experimental period, birds were caged and fed individually in triple-decked units equipped with individual feeders and waterers. In experiment 1, four cockerels were used per treatment; in other experiments, six cockerels were fed each experimental diet.

Experimental diets presented in tables 1, 2 and 3 were formulated and fed to provide the desired energy and protein restrictions without using large amounts of inert filler. Practical corn-soybean diets were fed in experiment 1, whereas semi-purified diets, based on glucose or sucrose and isolated soybean protein, were fed in experiments 2, 3 and 4.

TABLE 1

Diet composition (exp. 1)

Ingredient	Diet proportions		
	1 and 2 (basal)	3 and 4 (energy restricted)	5 and 6 (protein restricted)
	g	*g*	*g*
Corn, ground yellow	71.5	42.0	80.0
Soybean meal (50%)	18.0	23.0	6.5
Fish solubles, condensed	2.0	2.0	2.0
Alfalfa meal, dehydrated (20%)	2.0	2.0	2.0
Dicalcium phosphate	2.0	2.0	2.0
Oystershell flour	1.0	1.0	1.0
Premix C-20 [1]	1.0	1.0	1.0
Salt mix [2]	0.5	0.5	0.5
Cellulose or cholesterol [3]	2.0	2.0	2.0
Total [4]	100.0	75.5	97.0
Protein, %	17.1	17.0	12.0
Energy, ME [5] kcal/kg	2963	2075	2974

[1] Premix C-20 provides per kilogram diet: vitamin A, 7000 IU; vitamin D_3, 1000 ICU; vitamin E, 10 IU; and vitamin B_{12}, 10 μg; and (in milligrams) vitamin K, 1; riboflavin, 5; pantothenic acid, 10; niacin, 25; choline, 450; ethoxyquin, 125; penicillin, 6.6; and streptomycin, 33.
[2] Salt mix provides per kilogram of diet: NaCl, 4.25 g; and (in milligrams) Mn, 125; Zn, 3.3; Fe, 50; and Cu, 5.
[3] Two percent cholesterol replaced cellulose in diets 2, 4 and 6.
[4] Diets 3 and 4 fed at 75.5% rate of diets 1 and 2. Diets 5 and 6 fed at 97.0% rate of diets 1 and 2.
[5] Metabolizable energy.

TABLE 2

Diet composition (exp. 2)

Ingredient	Diet			
	1	2	3	4
	%	%	%	%
Glucose	56.22	61.47	66.47	71.72
Isolated soybean protein	27.00	21.75	16.75	11.50
Soybean oil	5.00	5.00	5.00	5.00
Cellulose	3.00	3.00	3.00	3.00
Cholesterol	2.00	2.00	2.00	2.00
Mineral mix [1]	4.78	4.78	4.78	4.78
Premix [2]	1.00	1.00	1.00	1.00
Methionine	0.67	0.67	0.67	0.67
Glycine	0.33	0.33	0.33	0.33
Total	100.00	100.00	100.00	100.00
Calculated to provide:				
Protein, %	22.1	18.0	14.1	10.0
Energy, ME kcal/kg	3538	3529	3520	3509

[1] Mineral mix provides per kilogram diet: (in grams) Ca, 11; P, 6; NaCl, 4.25; and K, 2; (in milligrams) Mn, 125; Zn, 51; Fe, 51; Cu, 5; and Mg, 660.
[2] Premix provides per kilogram diet: vitamin A, 7920 IU; vitamin D_3, 594 ICU; vitamin E, 31 IU; (in milligrams) vitamin K, 2.2; thiamine, 6.6; riboflavin, 4.4; pantothenic acid, 13.9; niacin, 39.6; pyridoxine, 4.4; biotin, 0.13; folic acid, 0.88; *p*-aminobenzoic acid, 110; and ethoxyquin, 125; and inositol, 1.1 g; choline chloride, 1.87 g; and vitamin B_{12}, 13 μg.

TABLE 3

Diet composition (exps. 3 and 4)

Ingredient	Diet proportions				
	Exp. 3		Exp. 4		
	1 and 2 (basal)	3 and 4 (energy restricted)	1 and 2 (basal)	3 and 4 (energy restricted)	5 and 6 (protein restricted)
	g	*g*	*g*	*g*	*g*
Glucose	72.72	47.72	—	—	—
Sucrose	—	—	50.44	25.44	58.44
Isolated soybean protein	11.50	11.50	20.00	20.00	12.50
Soybean oil	5.00	5.00	5.00	5.00	5.00
Cellulose [1]	4.00	4.00	4.00	4.00	4.00
Mineral mix [2]	4.78	4.78	4.56	4.56	4.56
Premix [3]	1.00	1.00	1.00	1.00	1.00
Methionine	0.67	0.67	0.67	0.67	0.67
Glycine	0.33	0.33	0.33	0.33	0.33
Total [4]	100.00	75.00	86.00	61.00	86.50

[1] Cholesterol replaced 1% cellulose in diets 1 and 3 in experiment 3, and in diets 2, 4 and 6 in experiment 4.
[2] Mineral mix provided per kilogram diet the same composition as listed in table 2, footnote 1.
[3] Premix provided per kilogram diet the same composition as listed in table 2, footnote 2.
[4] Experiment 3, diets 3 and 4 fed at 75% rate of diets 1 and 2. Experiment 4, fed at scheduled amounts of 86, 61 and 86.5% of consumption of similar aged, full-fed cockerels in previous experiments.

In experiments 1 and 3, birds receiving restricted diets were fed a quantity of feed determined by the amount of feed consumed by the birds fed the basal diet, expressed as a percentage of body weight. For experiment 2, isocaloric diets were fed ad libitum. In experiment 4, it was desirable to keep soybean oil and cellulose levels similar to those in previous experiments and still obtain the desired caloric content in the basal diet. This necessitated feeding an amount of feed, expressed as a percentage of body weight, according to a regimen calculated from feed consumption of cockerels of similar age in three previous experiments.

Feed allotments for each bird were weighed daily in experiments 1, 3 and 4, according to feeding regimens calculated each week from body weight and feed consumption data.

Birds were fasted 12 hours before blood samples were obtained from a brachial vein. Total serum cholesterol was determined by an automated colorimetric procedure.[4] A simple turbidimetric method was used for total lipid determinations (17).

Liver lipids were extracted on a Goldfisch extraction apparatus with a solvent mixture containing ethyl alcohol, ethyl ether and Skellysolve-B, in a 5:5:1 proportion.

Aortas were dissected from the point where the aorta enters the heart to just below the iliac bifurcation, stripped of adhering tissues, and separated into thoracic and abdominal segments. After staining, each segment was visually scored 1 to 4 by three individuals according to the amount of lipid stain and severity of lesions present.

Systolic blood pressure was measured indirectly as described by Nichols et al. (18).

A completely randomized design was used in experiments 1, 2 and 3, whereas a randomized block design was used for experiment 4 (19). A least squares analysis of variance designed for data with unequal subclass numbers (20) was used for experiments 1, 2 and 3; experiment 4 was analyzed by general analysis of variance. Duncan's multiple range test was used where appropriate.

RESULTS

Experiment 1. Practical diets with and without added cholesterol were fed to 5-week-old cockerels for 12 weeks. Protein or energy intake was restricted to approximately 70% of that provided by the basal diet.

[4] Technicon Instruments Corporation 1964 Total cholesterol. Technicon Auto-Analyzer Methodology-Method File N-24, Technicon Instruments Corporation, Chauncy, N. Y.

Body weight gains were greatly reduced when protein or energy intake was reduced (table 4). Although birds fed the nutrient-restricted diets had lower systolic blood pressures, differences were significant ($P < 0.05$ and $P < 0.01$) only for birds fed energy-restricted diets without and with added cholesterol, respectively.

Added dietary cholesterol increased ($P < 0.05$) serum cholesterol in birds fed basal and protein-restricted diet and, to a lesser extent, in birds fed the energy-restricted diet (fig. 1). Serum cholesterol was reduced ($P < 0.05$) when energy intake was restricted, and was increased when protein intake was restricted in birds fed diets containing added cholesterol. Similar effects, but less pronounced and nonsignificant, resulted when cholesterol was not added to the diets.

Liver lipids were increased when diets contained added cholesterol. Protein restriction, with and without added cholesterol, increased liver lipid concentration, whereas energy restriction resulted in only slight changes in liver lipid concentration.

Figure 2 illustrates the incidence of atherosclerosis in this experiment. Thoracic and abdominal aorta scores were not significantly affected by protein or energy restriction when diets did not contain added cholesterol. Scores of both thoracic and abdominal aortas were increased significantly ($P < 0.05$ and $P < 0.01$) by the addition of cholesterol to basal and protein-restricted diets, respectively. When comparing birds fed diets with added cholesterol, energy restriction tended to reduce, and protein restriction to increase, thoracic aorta scores.

Experiment 2. Isocaloric, semipurified diets containing 2% cholesterol and 22, 18, 14 or 10% protein were fed to 9-week-old cockerels. Average daily protein intakes for the 10-week experimental period ranged from 10.3 to 5.5 g/day per kilogram body weight (table 5).

Weight gains decreased progressively as protein intake was reduced. Blood pressure was not significantly affected. Serum cholesterol levels were significantly greater ($P < 0.05$) for birds when protein intake was restricted to 6.5 or 5.5 g/day per kilogram body weight. The range of protein levels used did not markedly affect the incidence of atherosclerosis in either aorta segment over the 10-week experimental period.

Experiment 3. Isonitrogenous, 10% protein, semipurified diets containing 3520 or 2640 metabolizable energy (ME) kcal/kg were fed with or without 1% added cholesterol to 9-week-old cockerels. Data from this 6-month experiment are presented in table 6. Weight gains were reduced when calorie intake was reduced and when diets were supplemented with cholesterol. Mean weight gains of birds fed the lower energy, cholesterol-containing diet were very low primarily because of weight losses during the last 8 weeks of the experiment. Birds from this treatment had lower blood pressures than did birds on other treatments, but the differences were not significant at the 5% level.

TABLE 4

Effect of restricted protein or energy intake on cockerels fed practical diets with and without added cholesterol

Treatment	Avg data (12-week period)			
	Body wt gain	Systolic blood pressure	Serum cholesterol	Liver lipid
	kg	*mm Hg*	*mg/100 ml*	*%* [1]
Basal	2.31	146	146	15.0 ± 1.96
Energy restricted [2]	0.96	126	131	16.4 ± 1.87
Protein restricted	1.17	129	160	20.6 ± 3.39
Basal + cholesterol [3]	2.20	147	366	26.3 ± 6.91
Energy restricted + cholesterol	1.01	115	206	24.0 ± 4.56
Protein restricted + cholesterol	1.11	133	483	36.0 ± 7.08

[1] Dry weight basis.
[2] Approximately 30% restriction for specified nutrient.
[3] Two percent cholesterol.

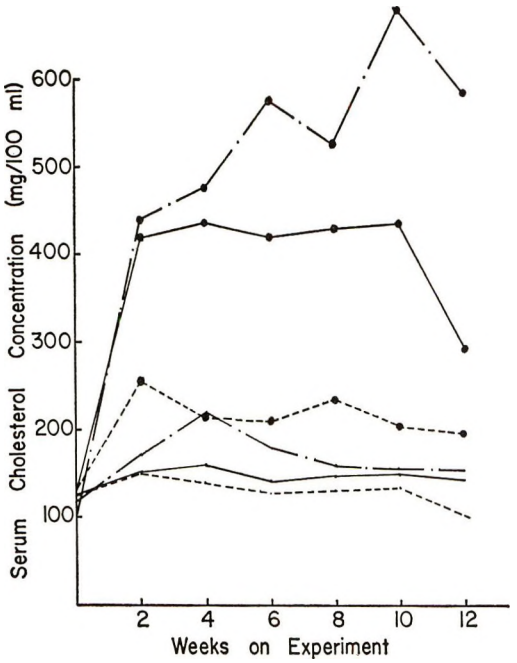

Fig. 1 Effect of energy or protein restriction on serum cholesterol levels of cockerels fed practical diets with and without added cholesterol: —— basal; - - - - - energy restricted; ——— · ——— protein restricted. Diets with 2% added cholesterol: ●———● basal; ●- - - - -● energy restricted; ●——— · ———● protein restricted.

Serum cholesterol and serum lipids were markedly increased ($P < 0.01$) by added cholesterol at either energy level. Lower serum cholesterol values ($P < 0.01$) were obtained, and serum lipids were slightly reduced, when calorie intake was reduced. The levels of calorie intake in this experiment had no effect on serum cholesterol or serum lipids when cholesterol was not added to the diets.

Liver lipids were increased in birds fed both energy intake levels when cholesterol was added, but to a much greater extent for birds fed the high energy diet. Liver lipids were decreased about 43 and 6% for birds fed the lower energy diet, with or without added cholesterol, respectively.

Figure 3 shows the degree of atherosclerosis in cockerels fed low protein diets at two energy levels. Thoracic and abdominal aorta scores were much higher ($P < 0.01$) for birds fed cholesterol. The magnitude of increase for thoracic aortas, however, was much less for birds with the

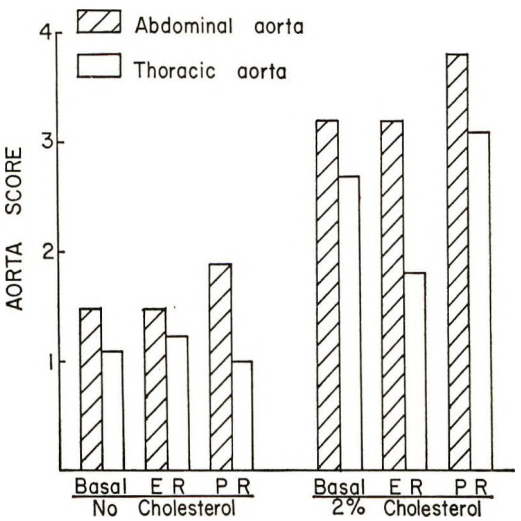

Fig. 2 Effect of energy or protein restriction on aorta scores of cockerels fed practical diets with and without 2% added cholesterol. ER (energy restricted); PR (protein restricted).

TABLE 5

Effect of varied protein intake on cockerels fed isocaloric, semipurified diets [1]

Protein level		Avg data (10-week period)				
Diet	Daily intake	Body wt gain	Systolic blood pressure	Serum cholesterol	Aorta score	
					A [2]	T [3]
%	*g/kg body wt per day*	*kg*	*mm Hg*	*mg/100 ml*		
22	10.3	2.05	159	380	2.6	2.3
18	8.5	1.98	160	434	2.2	2.5
14	6.5	1.84	170	570	2.7	2.3
10	5.5	1.62	163	543	2.7	2.7

[1] All diets contained 2% cholesterol.
[2] Abdominal aorta segment.
[3] Thoracic aorta segment.

TABLE 6

Influence of calorie intake on cockerels fed isonitrogenous, low protein [1] semipurified diets with and without added cholesterol

Treatment		Daily calorie intake,[4] ME	Avg data (24-week period)				
ME [2]	Added cholesterol [3]		Body wt gain	Systolic blood pressure	Serum cholesterol	Serum lipid	Liver lipid
kcal/kg		*kcal/kg body wt*	*kg*	*mm Hg*	*mg/100 ml*	*g/100 ml*	% [5]
3520	—	145	1.99	149	208	1.34	14.4 ± 2.39
2640	—	119	1.31	150	204	1.33	13.5 ± 1.71
3520	+	155	1.72	147	886	2.74	45.2 ± 6.03
2640	+	117	0.61	135	658	2.54	26.0 ± 6.38

[1] Ten percent protein.
[2] Metabolizable energy.
[3] One percent cholesterol.
[4] Average daily intake values for 24-week experimental period.
[5] Dry weight basis.

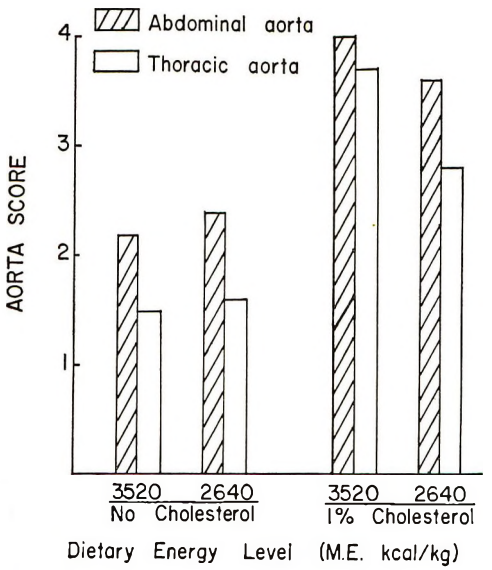

Fig. 3 Influence of lowered calorie intake on aorta scores of cockerels fed isonitrogenous (10% protein), semipurified diets with and without added cholesterol.

lower energy intake. Severity of aortic atherosclerosis was not influenced by energy intake differences in this experiment when diets did not contain added cholesterol. Aorta scores for birds fed high energy diets with added cholesterol were strikingly high. Aortas from this treatment were the most severely affected aortas noted in our laboratory to date.

Experiment 4. Energy and protein intakes averaging 73.5 and 65.0%, respectively, of the intakes of birds fed the basal diet were obtained for cockerels fed semipurified diets with and without added cholesterol. Data from this 12-week experiment (starting at 11 weeks of age) are given in table 7. Weight gains were reduced when any of the nutrient-restricted diets were fed, but to a much lesser extent in the protein-restricted group. Systolic blood pressure was higher ($P < 0.01$) in birds when protein intake was restricted and the diet contained added cholesterol. Energy restriction did not significantly affect blood pressure.

Cholesterol supplementation of all diets resulted in increased serum cholesterol ($P < 0.01$). The high serum cholesterol followed the pattern noted in previous experiments. Restricted energy intake reduced, and reduced protein intake increased, serum cholesterol concentrations when the diet contained added cholesterol. These relationships were significant ($P < 0.01$). Serum lipid responses to dietary treatments were similar to those described for serum cholesterol, except that serum lipids were also increased by protein restriction when diets did not contain added cholesterol.

Liver lipids were increased when cholesterol was added to the diet, but the increase was less for energy-restricted birds and greater for protein-restricted birds. In comparison with the appropriate basal diets, liver lipids of energy-restricted birds were decreased 31% when diets contained added cholesterol, and 8% when diets did not contain added cholesterol. Liver lipids, however, increased 18% in protein-re-

TABLE 7

Effect of restricted energy or protein intake on cockerels fed semipurified diets with and without added cholesterol

Treatment	Avg data (12-week period)						
	Body wt gain	Systolic blood pressure	Serum cholesterol	Serum lipid	Liver lipid	Aorta score	
						A [1]	T [2]
	kg	*mm Hg*	*mg/100 ml*	*g/100 ml*	*%* [3]		
Basal	1.62	131	178	1.20	17.7±2.6	2.1	1.1
Energy restricted [4]	0.46	123	200	1.27	16.3±2.5	2.8	1.2
Protein restricted	1.43	139	200	1.43	20.9±3.2	2.3	1.1
Basal + cholesterol [5]	1.77	124	438	1.93	34.6±7.6	3.9	3.3
Energy restricted + cholesterol	0.45	125	305	1.56	24.0±2.7	3.0	2.1
Protein restricted + cholesterol	1.48	144	770	2.40	40.7±2.8	3.7	3.6

[1] Abdominal aorta segment.
[2] Thoracic aorta segment.
[3] Dry weight basis.
[4] Twenty-six percent energy and 35% protein restrictions were obtained for the energy- and protein-restricted diets, respectively.
[5] One percent cholesterol.

stricted birds whether or not cholesterol was added to the diet.

When cholesterol was added to the diet, atherosclerosis was more prevalent ($P < 0.01$) in thoracic and abdominal aorta sections, with the exception of abdominal aortas from energy-restricted birds. Aorta scores were not significantly different when diets did not contain cholesterol. Energy restriction, however, resulted in significantly lower scores for thoracic aortas when diets contained added cholesterol.

DISCUSSION

As expected, restriction of dietary energy or protein intake reduced weight gains of cockerels. Energy restriction consistently resulted in lower weight gains than did comparable protein restriction. In experiment 4 (in which daily calorie intake average was 74% of that of birds fed the basal diet) body weights were barely maintained, and further energy restriction would have resulted in continued weight loss. In the same experiment, however, body weights were readily maintained by birds whose protein intake amounted to 65% of the protein consumed by birds receiving the basal diet. With this protein restriction, body weight gains were reduced only about 12 to 16%.

Overall, the effect of dietary energy or protein restriction on systolic blood pressure of cockerels appears inconsistent. This agrees with the results reported by Nichols and Balloun (3), that dietary restriction had no significant effect on systolic blood pressure of cockerels, although blood pressure was consistently lower for birds fed a restricted-energy diet. Speers [5] reported that different dietary protein or energy levels affected systolic blood pressure inconsistently in laying hens. The energy and protein effects detected, however, were of the same nature as in the present study.

Most of the blood pressure differences in the present experiments were probably related to the lighter body weights of birds fed diets with nutrient restriction, since it has been reported that systolic blood pressure of cockerels is directly associated with body weight changes (21). The lower blood pressures observed for birds fed energy-restricted diets containing added cholesterol in both experiments 1 and 3 cannot be explained entirely on the basis of body weight differences. However, this effect did not occur in experiment 4. A trend toward increased blood pressures for birds consuming less protein was observed in experiment 4 and, for one treatment, in experiment 2. This trend was evident even though the birds involved were slightly lighter than the birds fed the basal diet. The experiments reported here do not substantiate the conclusion of Nichols et

[5] G. M. Speers 1965 Effect of protein and energy on blood pressure and atherosclerosis in the laying hen. M. S. thesis, Iowa State University of Science and Technology, Ames, Iowa.

al. (18) that dietary cholesterol increases systolic blood pressure in cockerels.

Serum cholesterol concentration was increased in birds fed diets containing cholesterol, regardless of the nature of the diet. Restriction of energy or protein intake resulted in opposite responses of serum cholesterol concentration for birds fed cholesterol-supplemented diets.

Low protein intake markedly increased serum cholesterol. This effect of decreased protein intake was greater when semipurified diets were fed than when practical diets were fed.

On the other hand, low energy intake resulted in decreased serum cholesterol levels. The magnitude of the serum cholesterol response to energy restriction was not as great as that caused by protein restriction. Greater increases in serum cholesterol concentration were caused by added dietary cholesterol than by reduced protein. In most instances, the effect of restricted energy or protein intake on serum cholesterol concentration was not evident unless the diets contained added cholesterol. In a study with young chicks (1 to 5 weeks of age), however, Nishida et al. (9) reported that serum cholesterol levels were more dependent on dietary protein than on dietary cholesterol.

Total serum lipid concentration responded to dietary treatments much like serum cholesterol concentrations, with the exception that significantly higher serum lipid levels were obtained from birds fed a protein-restricted diet without added cholesterol.

Liver lipids increased when diets contained added cholesterol. With cholesterol-supplemented diets, protein restriction increased and energy restriction decreased liver lipids, following a response pattern similar to that of serum cholesterol and serum lipids for these same diets. When diets contained no added cholesterol, liver lipids were consistently slightly higher for birds fed protein-restricted diets.

Although atherogenic diets increased the incidence of atherosclerosis for thoracic and abdominal aortas, this incidence was more severe in abdominal segments than in thoracic segments, regardless of dietary treatment. The greater incidence of atherosclerosis in the abdominal aorta compared with that found in the thoracic aorta also has been noted in other studies (12). Another difference between thoracic and abdominal aorta sections was the nature of the atherosclerotic lesion. In most instances, the abdominal aortic lesion was in the form of thickened plaques, while the thoracic aortic lesion was more diffuse over the surface of the tissue, with plaques being formed in only two severe cases.

When diets did not contain added cholesterol, aorta score differences due to treatments were not significant. The results of Griminger et al. (1), showing a lower incidence of spontaneous atherosclerosis for birds fed 80% of the amount of food consumed by full-fed birds, may differ from those of the present study because of the length of their experimental period (37.5 months).

From the results of this study, it appears that cholesterol-induced atherosclerosis of the thoracic aorta is more readily affected by restricted energy intake than is atherosclerosis of the abdominal aorta.

Further studies utilizing more detailed biochemical analyses are needed to evaluate the relationships between dietary energy restriction per se and lipid metabolism and aortic atherosclerosis in cockerels. The effect of dietary energy restriction on the metabolism of cholesterol in the liver (including conversion to bile acids) should be given special attention.

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Effect of Ambient Temperature and Dietary Amino Acids on Carcass Fat Deposition in Rats

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ABSTRACT Sixty-three rats of the Sprague-Dawley strain were used in each of two experiments to study the effects of ambient temperature (7, 23 and 33°) on diet utilization. In experiment 1, dietary protein levels and protein-amino acid sources were: A) 31.7%, casein; B) 15.85%, casein; and C) 31.7%, diet B plus casein-simulating crystalline amino acid mixture. The rats, average initial weight of 122 g, were confined to individual metabolism cages and fed ad libitum for 20 days, then killed for carcass analysis. Diet intake increased significantly with decrease in temperature. Gain was highest at 23°, lowest at 7°. Gain-to-feed ratio results were similar at 23 and 33°, with both significantly higher than at 7°. Carcass fat decreased significantly as temperature decreased. For experiment 2, diet C of experiment 1 served as the control and the effects of deletions of certain of the crystalline amino acids were studied. With isonitrogenous diets, omission of the crystalline essential amino acids or of only the basic amino acids increased fat deposition. Carcass fat deposition, however, responded more to ambient temperature than to amino acid imbalance.

Many experiments have demonstrated that high levels of dietary crude protein decrease carcass fat deposition and that exposure of animals to cold environments increases feed intake. There is little information, however, on the effect of ambient temperature on carcass fat deposition. Payne and Jacob (1) calculated carcass fat content of 30-day-old rats exposed to environmental temperatures of 15 or 27° and fed either nitrogen-free, 4, 10 or 25% casein diets. Carcass fat was less at 15° than at 27°, except for the rats on the 4% casein diet. Schmidt and Widdowson (2) fed 10-week-old rats for 9 weeks a diet containing either 17 or 4.3% protein. Half the animals on each diet were kept at 21°, and half at 5°. Animals fed the 17% protein diet at 21° had the most carcass fat, but there were no significant differences among the other three groups. Bobek and Ginter (3) observed that esterified fatty acid was decreased and glycogen was increased in the liver when rats at 2° were fed high fat, high cholesterol diets. They assumed this effect was probably due to a lowering of hepatic lipogenesis. Young and Cook (4) exposed mice to ambient temperatures of 4, 24 and 35° for 30 to 36 days. The mice exposed to the low temperature consumed more food than those at 24°, failed to grow, and deposited less total lipid in the carcass than the control group at 24°. On the other hand, the mice exposed to the high temperature consumed less food than the controls (24°), grew as well as the control group, and deposited similar quantities of lipid. Heroux and Gridgeman (5) kept rats for 4 weeks in a warm (30°) or a cold (6°) environment. Weight of superficial fat tissue of the former was twice as much as that of the latter. Barnett and Widdowson (6), however, reported that male offspring from mice representing the 14th generation of continuous exposure to − 3° deposited more fat than those of controls at 21°. However, "new mice" exposed to − 3° for the first time deposited less fat than controls at 21°. Mickelberry et al. (7) observed that elevated temperature markedly retarded growth and feed consumption of chicks, but had no significant influence upon either the moisture or fat content of chick tissues.

The experiments reported here were conducted to study the effect of ambient temperature on carcass fat deposition in rats fed different levels and sources of dietary amino acids.

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EXPERIMENTAL

Experiment 1. Sixty-three male rats of the Sprague-Dawley strain, averaging 122 g, were randomly assigned to diets (A, B and C shown in table 1) and environments (7, 23 and 33°). Each rat was confined to a metabolism cage and diet and water given ad libitum for 20 days. Fresh feed was added daily in response to level of voluntary consumption. Prior to feeding, diets were stored in the 7° environment. Chamber temperatures were maintained within ± 0.5°, and relative humidity was kept constant at 50%. At the beginning of the experiment five representative rats were killed by ether asphyxia for carcass analysis. At the end of the experiment rats were killed by ether asphyxia and the entire carcass frozen for analysis. The frozen carcasses of seven rats from a treatment were ground and mixed, and from this homogeneous mass, samples were taken for chemical analysis. Moisture was determined by overnight drying in a vacuum oven at 90°. The dry sample was then transferred to an extraction thimble for a 16-hour ether extraction for determination of crude fat. Nitrogen was determined by the Kjeldahl method. Feces were collected during the experiment and fecal nitrogen was also determined.

TABLE 1

Composition of test diets (exp. 1)

Ingredients	A	B	C
	g/100 g diet		
Cornstarch	45.00	62.50	40.88
Casein	35.00	17.50	17.50
Amino acid mix [1]	—	—	20.62
Corn oil	10.00	10.00	10.00
Cellulose	2.00	2.00	2.00
Mineral mix [2]	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
$NaHCO_3$	—	—	1.00
Vitamin mix [3]	1.00	1.00	1.00
Cod liver oil	1.50	1.50	1.50
Wheat germ oil	0.50	0.50	0.50
Crude protein, % [4]	31.70	15.85	31.70

[1] Composition is shown in table 2.

[2] Mixture contained: (in milligrams) NaCl, 108,088; $K_2C_6H_5O \cdot H_2O$, 236,888; K_2HPO_4, 77,332; $CaHPO_4 \cdot 2H_2O$, 335,554; $CaCO_3$, 163,554; $MgCO_3$, 40,888; $FeC_6H_5O_7 \cdot 3H_2O$, 15,998; $CuSO_4 \cdot 5H_2O$, 178; $MnSO_4$, 1,245; $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 88; KI, 44; $ZnCO_3$, 441.

[3] One hundred-gram mixture contained: (in milligrams) Thiamine·HCl, 26; riboflavin, 66; nicotinic acid, 440; pantothenic acid, 264; pyridoxine, 25; choline chloride, 22,000; vitamin B_{12}, 0.44; vitamin K (menadione), 2; vitamin E (γ-tocopherol), 1,200; folic acid, 20; biotin, 2; ascorbic acid, 3,000; and finely powdered starch to 100 g.

[4] Includes assay value for casein and calculated contribution of crystalline amino acids.

Experiment 2. Sixty-three male rats of the Sprague-Dawley strain, averaging 90 g, were confined to an environment of 33° for 21 days. Diet C from experiment 1 served as a control diet. The amino acid composition of experimental diets is shown in table 2. Treatment of rats and analyses procedures were the same as described in experiment 1.

RESULTS

Experiment 1. Feed consumption, weight gain and gain-to-feed ratio are shown in table 3. Feed intake of all diets decreased significantly ($P < 0.01$) at 33° and increased significantly ($P < 0.01$) at 7° when compared with a room temperature of 23°. Within each environment feed intake did not differ significantly among diets. Growth rate on all diets decreased at both high and low temperatures, but cold exposure decreased growth rate more than did heat exposure, except on diet B. Diet A produced significantly higher gains at 23° ($P < 0.05$) and at 33° ($P < 0.001$) than did diet B. The latter was not significantly better than diet C. At 7°, however, gains were similar on diets A and B, and lowest on diet C. Gain-to-feed ratios on the respective diets were similar at 23 and 33°. At 7°, the values were significantly ($P < 0.05$) lower. On the average, diet A gave significantly ($P < 0.05$) higher gain to feed than did the other two diets.

Carcass composition data are shown in table 4. It was clear that temperature significantly ($P < 0.01$) affected carcass fat deposition. Percentage crude fat was significantly ($P < 0.05$) less at 7° than at 23°. The trend toward increased fat deposition at high temperature was greatest on the lower protein diet (diet B), while the depressing effect of low temperature was greatest on the higher protein diet (diet A). Also, the crystalline amino acid mixture stimulated fat deposition more than intact protein in all tempreatures (diet C versus diet A). Temperature significantly affected carcass moisture ($P < 0.01$) and crude protein ($P < 0.05$), values increasing as temperature decreased.

TABLE 2

Composition of test diets (exp. 2)

Ingredient [1]	Diet description								
	C	D	E	F	G	H	I	J	K
	%	%	%	%	%	%	%	%	%
Cornstarch	40.88	39.16	34.86	38.96	42.17	40.94	41.41	38.37	40.97
Casein	17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50	17.50
Amino acid mix	20.62	22.34	26.64	22.54	19.33	20.56	20.09	23.13	20.53
L-Arginine·HCl	0.91	0.91	—	—	1.55	0.91	0.91	—	0.91
L-Histidine·HCl·H_2O	0.65	0.65	—	—	1.11	0.65	0.65	—	0.65
L-Lysine·HCl	1.89	1.89	—	—	3.22	1.89	1.89	—	1.89
L-Tryptophan	0.27	0.27	—	—	0.46	0.27	—	0.27	0.27
L-Isoleucine	1.13	1.13	—	—	1.93	—	1.13	1.13	1.13
L-Valine	1.30	1.30	—	—	2.22	—	1.30	1.30	1.30
L-Leucine	1.68	1.68	—	—	2.86	—	1.68	1.68	1.68
L-Threonine	0.82	0.82	—	—	1.40	—	0.82	0.82	0.82
L-Methionine	0.53	0.53	—	—	0.90	0.53	0.53	0.53	—
L-Cystine	0.06	0.06	—	—	0.10	0.06	0.06	0.06	—
L-Phenylalanine	0.99	0.99	—	—	1.69	0.99	—	0.99	0.99
L-Tyrosine	1.11	1.11	—	—	1.89	1.11	—	1.11	1.11
L-Glutamic	4.02	11.00	26.64	9.76	—	6.13	4.82	6.60	4.24
L-Aspartic	1.34	—	—	3.35	—	2.04	1.61	2.20	1.41
L-Proline	2.09	—	—	5.08	—	3.19	2.50	3.43	2.20
L-Serine	1.07	—	—	2.60	—	1.63	1.28	1.76	1.13
L-Alanine	0.51	—	—	1.24	—	0.78	0.61	0.84	0.54
Glycine	0.25	—	—	0.61	—	0.38	0.30	0.41	0.26

[1] Other ingredients were the same as shown in table 1. All diets were isonitrogenous. L-Cystine and L-tyrosine were included in essential amino acids.

TABLE 3

Feed consumption, weight gain and gain-to-feed ratio of rats (exp. 1)

	Temperature			Avg
	33°	23°	7°	
Avg daily feed consumed, g [1]				
Diet A	10.0 ±0.37 [2]	13.4 ±0.57	17.9 ±1.13	13.8
Diet B	9.6 ±1.30	13.2 ±0.92	18.4 ±0.95	13.7
Diet C	9.5 ±0.52	12.5 ±0.65	17.4 ±0.76	13.1
Avg [3]	9.7	13.0	17.9	
Avg daily gain, g				
Diet A	3.9 ±0.27	5.2 ±0.57	3.2 ±0.60	4.1 [4]
Diet B	3.3 ±0.74	4.4 ±0.98	3.3 ±0.74	3.7
Diet C	3.1 ±0.27	4.1 ±1.15	2.6 ±0.57	3.3
Avg [3]	3.4	4.6	3.0	
Gain/feed				
Diet A	0.38±0.02	0.39±0.04	0.18±0.03	0.32 [5]
Diet B	0.34±0.04	0.33±0.01	0.18±0.03	0.28
Diet C	0.32±0.03	0.33±0.03	0.15±0.03	0.27
Avg [6]	0.35	0.35	0.17	

[1] Values represent means of seven rats fed the experimental diets for 20 days.
[2] SEM.
[3] Twenty-three degrees significantly ($P < 0.01$) over 33°; significantly ($P < 0.001$) less than 7°.
[4] Diet A significantly higher than B at 23° ($P < 0.05$) and 33° ($P < 0.001$).
[5] $P < 0.05$.
[6] $P < 0.001$.

The retention of nitrogen and energy, and the efficiency of nitrogen and energy are shown in table 5. Cold exposure significantly ($P < 0.01$) decreased nitrogen efficiency regardless of diet. But the depression occurring in rats fed diet B was appreciably smaller than for the other two diets. Nitrogen efficiency from diet B was

TABLE 4

Carcass composition of rats (exp. 1)

Item	Temperature			Avg
	33°	23°	7°	
Crude fat, %				
Diet A	11.2	10.7	6.8	9.6
Diet B	15.2	10.6	8.4	11.4
Diet C	13.4	11.1	8.6	11.0
Avg [1]	13.3	10.8	7.9 [2]	
Moisture, %				
Diet A	65.5	67.0	69.6	67.4
Diet B	62.4	67.2	68.7	66.1
Diet C	63.1	65.6	68.6	65.8
Avg [1]	63.7	66.6	69.0	
Crude protein, %				
Diet A	20.1	20.8	21.3	20.7
Diet B	19.6	20.1	21.6	20.4
Diet C	19.1	21.1	20.5	20.2
Avg [3]	19.6	20.7	21.1	

[1] $P < 0.01$.
[2] $P < 0.05$, 23° over 7°.
[3] $P < 0.05$.

TABLE 5

Nitrogen and energy retention (exp. 1)

	Temperature			Avg
	33°	23°	7°	
Nitrogen retention, g/rat [1]				
Diet A	2.7	3.7	2.6	3.0 [2]
Diet B	2.1	2.9	2.8	2.6
Diet C	2.0	3.1	1.9	2.3
Avg [2]	2.3	3.2	2.4	
Nitrogen efficiency, % [3]				
Diet A	27.2	28.6	15.2	23.7
Diet B	48.1	46.9	32.3	42.4 [4]
Diet C	21.1	25.3	11.3	19.2
Avg [5]	32.1	33.6	19.6	
Energy retention, kcal/rat [6]				
Diet A	231	274	139	215
Diet B	269	212	169	217
Diet C	232	245	141	206
Avg [2]	244	244	150	
Energy efficiency, % [7]				
Diet A	26.2	23.2	8.8	19.4
Diet B	31.7	19.3	10.4	20.5
Diet C	27.9	22.2	9.2	19.8
Avg [8]	28.6	21.6	9.5	

[1] Final carcass nitrogen — initial carcass nitrogen.
[2] $P < 0.05$.
[3] Nitrogen retained/nitrogen absorbed × 100.
[4] Diet B significantly higher ($P < 0.01$) than diets A and C.
[5] $P < 0.01$.
[6] (Fat retained (g) × 9.3) + (nitrogen retained (g) × 6.25 × 4.1).
[7] Calories retained/calories consumed × 100.
[8] Seven degrees significantly less ($P < 0.05$) than 23°.

significantly higher than for diet A ($P < 0.05$) and diet C ($P < 0.01$). Rats on diet B at 33° deposited more energy than those at 23°, although the growth rate at 33° was lower than at 23°. Higher temperature increased carcass fat deposition and energy efficiency on all diets although gain-to-feed ratios in both groups were the same. Energy efficiency at 7° was significantly lower ($P < 0.05$) than at 23°.

Experiment 2. Growth rates on diets C (control), D (crystalline NEAA [2] replaced by glutamic acid), H (omission of isoleucine, valine, leucine and threonine) and J (omission of basic amino acids) did not differ significantly. This was also true among diets F (omission of EAA [3]), G (omission on NEAA) and I (omission of tryptophan, phenylalanine and tyrosine). Diet K (omission of sulfur amino acids) resulted in the lowest feed consumption, growth rate and gain-to-feed ratio of all diets. Rats fed diet E (crystalline amino acid mixture replaced by glutamic acid) showed the least growth during week 1, but thereafter had reasonable growth rate (table 6).

Carcass composition and nitrogen efficiency are shown in table 7. Omission of all crystalline EAA (diet F) resulted in the greatest amount of carcass fat. Omission of basic amino acids (diet J) appeared to increase carcass fat deposition. Diets D (crystalline NEAA replaced by glutamic acid) and E (all crystalline amino acids replaced by glutamic acid) showed slight increase in carcass fat deposition. Rats fed diet G (omission of crystalline NEAA), however, had the lowest crude fat content. Nitrogen retention and efficiency were highest by rats fed diet C (control).

DISCUSSION

Rats at 23 and 33°, fed a 15.8% crude protein diet (diet B), grew slower than those fed a 31.7% crude protein diet (diet A), but at 7° growth rates were similar on both diets. The responses to temperatures reflect differences in feed intake. At 7° compensatory feed intake would overcome the possible deficiency of sulfur amino acids in the 15.8% protein diet. Payne

[2] NEAA, nonessential amino acids.
[3] EAA, essential amino acids.

TABLE 6

Feed consumption, weight gain and gain-to-feed ratio of rats (exp. 2) [1]

Diet	Feed consumed [2]	Weight gain [2]	Gain/feed [2]
	g/rat/day	*g/rat/day*	
C (control)	7.3 ± 0.43 [3]	3.0 ± 0.32	0.41 ± 0.03
D (EAA + Glu)	7.2 ± 0.25	2.8 ± 0.37	0.39 ± 0.04
H (— Ile, Val, Leu, Thr)	7.4 ± 0.64	3.1 ± 0.47	0.41 ± 0.04
J (— Arg, His, Lys)	7.1 ± 0.38	2.8 ± 0.40	0.40 ± 0.04
F (— EAA)	6.3 ± 0.50	2.1 ± 0.41	0.32 ± 0.04
G (— NEAA)	6.1 ± 0.34	2.1 ± 0.45	0.34 ± 0.06
I (— Trp, Phe, Tyr)	6.7 ± 0.24	2.3 ± 0.29	0.34 ± 0.04
E (all Glu)	6.2 ± 0.25	1.7 ± 0.25	0.27 ± 0.04
K (— Met, Cys)	5.8 ± 0.72	1.2 ± 0.45	0.20 ± 0.07

[1] Values represent means of seven rats fed the experimental diets for 21 days.
[2] $P < 0.001$.
[3] SEM.

TABLE 7

Carcass composition and nitrogen efficiency of rats (exp. 2)

Diet	Composition of fresh carcass			Nitrogen retained [1]	Nitrogen efficiency [1]
	Moisture	Crude fat	Crude protein		
	%	%	%	*g/rat*	%
C (control)	67.6	8.0	20.5	2.4	31.6
D (EAA+Glu)	68.2	8.5	20.1	2.1	29.1
H (— Ile, Val, Leu, Thr)	67.2	8.2	19.7	2.2	29.3
J (— Arg, His, Lys)	67.1	9.0	20.3	2.2	30.6
F (— EAA)	67.6	9.8	19.3	1.5	21.0
G (— NEAA)	67.8	7.6	20.5	1.8	28.4
I (— Trp, Phe, Tyr)	67.6	7.8	20.0	1.8	26.3
E (all Glu)	68.3	8.6	19.6	1.3	19.9
K (— Met, Cys)	68.6	7.8	20.0	1.0	17.7

[1] Shown in table 5.

and Jacob (1) observed that rats fed a 4% casein diet grew slightly better, and those fed a 10% casein diet markedly better, at 15° than those fed the same diets at 27°. In contrast, rats fed a 25% casein diet grew more slowly at the lower temperature. Schmidt and Widdowson (2) reported that rats eating a stock diet (crude protein 17%) and kept at 5° consumed more feed but grew less than those kept at 21°. When fed a low protein diet (crude protein 4.3%) rats at 21° lost weight, whereas those at 5° consumed more feed and lost weight only slightly during the first 3 weeks, and thereafter maintained body weight for 9 weeks. Treadwell et al. (8) also noted better growth rates at 1° than at 25° by rats receiving diets of 5 and 10% protein. However, performance on diets having greater than 10% protein was lower at the cold environment. Similar results were described by Meyer and Hargus (9). Since cold exposure stimulates feed intake, a diet deficient in a given nutrient for animals in a warm environment would, in a cold environment, be consumed in amounts sufficient to provide more than adequate absolute intake of the nutrient in question. Borchers (10) reported that growth inhibition of raw soybean meal was less for rats at 10° than at 30°. This alleviating effect of low temperature appears to reflect increased feed intake, since the supplemental combination of DL-methionine and DL-valine was effective in alleviating the growth inhibition effect of raw soybean meal fed to rats at 30°. Similar effects of cold exposure due to increased feed intake were reported by Klain et al. (11), Bavetta and Nimni (12) and Harper and Rogers (13).

High ambient temperature increased carcass fat deposition, especially in rats fed the low protein diet. The lower temperature decreased carcass fat deposition and it was most apparent with the high pro-

tein diet. It would appear that at the high temperature feed intake was not sufficiently reduced to prevent excess calorie consumption and fat deposition. Conversely, at the low temperature, intake was insufficient to compensate for the added energy expenditure in thermogenesis and body heat loss. Moreover, the stress caused by ambient temperature change may have altered some conditions of energy (hence protein) metabolism in the rat, and this alteration was affected by dietary crude protein content.

Rats fed diet C (combination of casein and crystalline amino acids) showed poorer growth rate and deposited more carcass fat than those fed diet A (all amino acids from casein) in all temperatures, although both diets had the same crude protein content and amino acid balance. This indicates that the crystalline amino acid mixture, designed to duplicate the amino acid balance of casein, had nutritional characteristics different from intact protein. Perhaps the rate and pattern of amino acid absorption from the gastrointestinal tract differed sufficiently between intact protein and crystalline amino acids to affect metabolic behavior.

In experiment 2, the effects of deletions of certain of the crystalline amino acids were studied. It was found that isonitrogenous diets containing different amino acid patterns differentially affected carcass fat deposition. Omission of crystalline EAA (diet F) caused the greatest, and omission of crystalline NEAA (diet G) the least, amount of carcass fat deposition. Diet J (omission of crystalline basic amino acids) also increased carcass fat deposition, and diet H (omission of crystalline essential aliphatic amino acids) only slightly increased carcass fat deposition. Thus, the depressing effect on carcass fat deposition of essential amino acids may be due mainly to the basic amino acids. On the other hand, diet F increased carcass fat deposition and this showed that the crystalline NEAA increased carcass fat deposition. Diet D (crystalline NEAA mixture replaced by glutamic acid) and diet E (crystalline amino acid mixture replaced by glutamic acid) also increased carcass fat deposition. Thus, glutamic acid stimulated greater fat deposition than did the NEAA of diet C. It cannot be said, however, that glutamic acid has a greater effect on increasing carcass fat deposition than any of the individual NEAA, since these have not been evaluated individually.

It is suggested that the wide differences in growth rate among the test diets in experiment 2 resulted from amino acid imbalances. Rats fed diets C (control) and H had similar growth rates, but the former retained more nitrogen and showed better nitrogen efficiency than the latter. This would reflect the better balance of amino acid in diet C. Diet K (omission of crystalline sulfur amino acids) was consumed in least amounts and produced the lowest growth rate. This would appear to reflect the result of the severe amino acid imbalance due to the low content of sulfur amino acids. However, diets E and F, in which the sulfur amino acid content was the same as in diet K, showed appreciably better growth rate than diet K. It is likely that the presence of other crystalline EAA accentuated the amino acid imbalance of diet K.

In the two trials reported here it was found that high ambient temperature increased and low ambient temperature decreased carcass fat deposition. These temperature effects appeared to differ among dietary protein levels. Dietary amino acid balance also influenced carcass fat deposition in the rat. It was evident, however, that carcass fat deposition responded more to temperature than to the dietary amino acid balance.

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Influence of Zinc and Vitamin D on Plasma Amino Acids and Liver Xanthine Oxidase in Rats [1,2]

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ABSTRACT Plasma amino acids and liver xanthine oxidase activity were determined in rats fed a control diet with and without calciferol or fed diets providing 9% casein with and without added zinc and calciferol. Reduction of dietary protein resulted in a significant decrease in xanthine oxidase activity, concentrations of plasma threonine, methionine, isoleucine, leucine and phenylalanine, and in elevated histidine levels. Addition of calciferol to diets containing added zinc decreased the levels of plasma isoleucine, leucine, threonine and lysine. Lysine in plasma was also decreased by calciferol supplementation of the low protein diet and by the addition of zinc to this diet. Xanthine oxidase activity was lowest in animals fed the low protein diet without calciferol and zinc. The apparent interaction between zinc and vitamin D closely approached significance at the 5% level. The results indicated that xanthine oxidase activity in animals fed low protein decreased with increased plasma lysine, which was observed in animals maintained without dietary sources of zinc and calciferol.

A previous comparison of the concentrations of selected essential amino acids in plasma of rats fed at two levels of casein, with and without dietary calciferol, indicated that the amount of lysine in plasma was lower in animals fed calciferol (1). Irregular appearance of fur of animals fed 9% casein with calciferol suggested that zinc deficiency may have contributed to the changes observed in ratios of plasma amino acids and in appearance of the animals. An evaluation of mineral mixtures used in experimental diets by Williams and Briggs (2) pointed out that zinc should be added to USP XIV salt mixture (3) which was used in this laboratory without added zinc.

The purpose of the present investigation was to compare plasma amino acid patterns in animals fed 9% casein with and without added zinc and calciferol, with those of control animals fed 18% casein plus zinc supplementation, with and without calciferol. Because studies have indicated that the activity of xanthine oxidase may be related to the level of dietary lysine (4, 5) and zinc (6), activity of liver xanthine oxidase was also investigated.

METHODS AND MATERIALS

Male rats of the Sprague-Dawley strain, weighing 40 to 60 g, were allocated to 6 groups of 12 each according to body weight and litter. The two control groups each received a vitamin D-deficient diet which provided 18% casein supplemented with 12 mg zinc/kg diet in the form of zinc carbonate to provide 22 mg zinc/kg diet. Two groups each received a vitamin D-deficient diet containing 9% casein supplemented with 12 mg zinc/kg to provide 20 mg zinc/kg diet, and two groups were each fed a similar diet without added zinc which provided 8 mg zinc/kg diet. The chemical method described by McCall et al. (7) was used to determine the zinc content of the diets. One group of 12 rats assigned to each diet received 10,000 IU vitamin D (calciferol) orally per week. The vitamin D supplement, which was administered orally three times per week, was prepared by the addition of a weighed quantity of calciferol to a weighed amount of cottonseed oil. The 12 corresponding animals assigned to each dietary treatment received a comparable amount of carrier without added calciferol. The composition of the diets is shown in table 1. The ani-

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TABLE 1

Composition of diets

	9% protein	18% protein
	%	%
Casein [1]	9	18
Sucrose	78	69
Cottonseed oil [2]	5	5
Salt mixture [3]	4	4
Cellulose [4]	4	4
		mg/kg
Thiamine·HCl		5.0
Pyridoxine·HCl		2.5
Riboflavin		8.0
Ca pantothenate		20.0
Niacin		10.0
Biotin		0.1
Folacin		2.0
Vitamin B_{12}		0.02
Inositol		100.0
p-Aminobenzoic acid		100.0
Menadione		0.5
Choline chloride		1300.0
Vitamins A and E [5]		

[1] Vitamin-free casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Wesson Oil, Wesson Sales Company, Fullerton, Calif.
[3] United States Pharmacopoeia, ed. 14. 1950, p. 789.
[4] Solka Floc, Brown Company, San Francisco, Calif.
[5] Cottonseed oil-vitamin D preparation provided 1000 IU vitamin A acetate and 0.8 mg α-tocopherol per rat per week.

mals were housed in individual galvanized metal cages with wire bottoms and given food and distilled water ad libitum during the 6-week experimental period.

An amino acid analyzer [3] was used to determine the concentration of amino acids in protein-free filtrates of blood plasma prepared from pooled plasma samples from four nonfasted rats (8). Activity of liver xanthine oxidase was estimated according to the method reported by Litwack et al. (9). Statistical measurements used to evaluate the results were analysis of variance and *t* test (10).

RESULTS

Rats fed the control diet with and without vitamin D supplement gained 226 ± 3 and 192 ± 11 g, respectively. The difference was significant ($P < 0.01$). Supplementation of the diet providing 9% casein with vitamin D or zinc had no apparent effect on appearance of fur or on weight gain. Rats fed 9% casein plus added zinc with and without vitamin D gained 89 ± 5 and 92 ± 3 g, respectively. Corresponding animals fed the same level of protein without supplementary zinc gained 92 ± 5 and 98 ± 5 g.

Mean values for plasma amino acids are shown in table 1. Since the method employed in preparation of the plasma extracts involved treatment with acid, the chromatographs showed no detectable tryptophan in the samples obtained from rats fed the various experimental diets. As determined by analysis of variance the concentrations of valine, arginine, cystine, tyrosine, aspartic acid, serine, proline, glutamic acid and glycine were not significantly affected by dietary treatment. Reduction of protein from 18 to 9% of the diet produced a significant decrease in concentrations of plasma threonine, methionine, isoleucine, leucine ($P < 0.01$) and phenylalanine ($P < 0.05$), and increased levels of plasma histidine ($P < 0.05$). Addition of vitamin D to diets providing 18 or 9% protein with zinc supplementation decreased the levels of plasma isoleucine and leucine ($P < 0.01$) and of threonine and lysine ($P < 0.05$). Addition of zinc to the low protein diet resulted in significant decreases in the concentration of free plasma lysine ($P < 0.01$) and alanine ($P < 0.05$). Supplementation of the 9% casein diet with vitamin D decreased the concentrations of plasma lysine ($P < 0.01$).

The mean activity values for xanthine oxidase expressed in micromoles of xanthine oxidized per hour per gram of liver are shown in table 2. Activity curves plotted from the mean values for residual xanthine at each time interval observed are shown in figure 1. Xanthine oxidase activity of the liver was decreased by reduction of the protein level of the diet ($P < 0.01$). Vitamin D supplementation resulted in decreased values for activity of xanthine oxidase in liver of rats fed diets containing added zinc, and increased activity of the enzyme in liver of animals fed the diet without added zinc. The differences were not significant. Expressed as micromoles of xanthine oxidized per hour per gram of liver the lowest value of 1.36 was observed in animals fed 9% casein in the absence of zinc and vitamin D. The activity curves

[3] Beckman Model 120B, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

TABLE 2

Plasma free amino acid levels of weanling rats fed the control diets and diets containing 9% casein with and without zinc and vitamin D

Amino acid	18% casein + zinc		9% casein + zinc		9% casein − zinc	
	− vitamin D	+ vitamin D	− vitamin D	+ vitamin D	− vitamin D	+ vitamin D
	μmoles/100 ml		*μmoles/100 ml*		*μmoles/100 ml*	
Threonine	60.4 ± 2.8 [1]	47.4 ± 2.8	43.9 ± 2.2	39.0 ± 3.4	47.5 ± 3.0	40.4 ± 5.8
Valine	23.9 ± 0.6	18.7 ± 1.5	20.1 ± 0.6	17.1 ± 1.8	19.3 ± 4.7	20.2 ± 2.2
Methionine	6.0 ± 0.2	5.7 ± 0.4	3.5 ± 0.4	3.3 ± 0.4	4.5 ± 0.3	4.8 ± 0.6
Isoleucine	11.3 ± 0.4	9.4 ± 0.6	9.1 ± 0.3	7.0 ± 0.8	10.2 ± 0.6	9.2 ± 0.9
Leucine	17.9 ± 0.2	13.1 ± 1.7	12.2 ± 1.4	9.8 ± 0.3	12.8 ± 0.4	13.3 ± 1.0
Phenylalanine	6.8 ± 0.9	6.0 ± 0.8	5.7 ± 0.3	4.2 ± 0.4	5.1 ± 0.6	6.3 ± 0.7
Lysine	49.1 ± 2.7	39.8 ± 1.3	48.9 ± 2.5	41.4 ± 2.4	67.6 ± 0.5	49.5 ± 5.7
Histidine	7.1 ± 0.6	5.6 ± 0.8	10.2 ± 1.6	8.2 ± 0.7	12.3 ± 0.6	9.4 ± 1.3
Arginine	13.4 ± 1.6	12.8 ± 1.1	17.3 ± 2.9	13.3 ± 0.6	16.4 ± 1.6	15.2 ± 1.4
Half-cystine	3.1 ± 0.5	2.5 ± 0.2	2.2 ± 0.2	2.8 ± 0.2	2.1 ± 0.4	2.8 ± 0.4
Tyrosine	10.6 ± 0.6	10.3 ± 0.4	8.4 ± 2.6	7.6 ± 1.1	12.1 ± 1.1	9.8 ± 1.0
Aspartic acid	4.6 ± 1.8	2.5 ± 0.2	2.8 ± 0.6	3.1 ± 0.3	3.5 ± 0.1	3.0 ± 0.5
Serine	50.8 ± 8.6	53.7 ± 15.0	52.6 ± 8.8	63.8 ± 3.3	65.7 ± 1.4	84.8 ± 14.8
Proline	48.4 ± 4.1	34.7 ± 4.5	41.4 ± 13.7	35.1 ± 7.8	50.6 ± 6.0	43.6 ± 13.5
Glutamic acid	16.4 ± 0.3	17.0 ± 0.3	16.8 ± 3.2	21.2 ± 2.5	19.8 ± 0.8	20.4 ± 1.3
Glycine	31.4 ± 2.2	27.8 ± 2.2	38.2 ± 2.8	34.8 ± 2.5	35.3 ± 1.0	39.0 ± 2.9
Alanine	58.6 ± 1.4	47.9 ± 1.7	53.1 ± 9.3	60.5 ± 4.7	71.5 ± 6.4	65.5 ± 4.3

[1] Mean of three samples ± SEM.

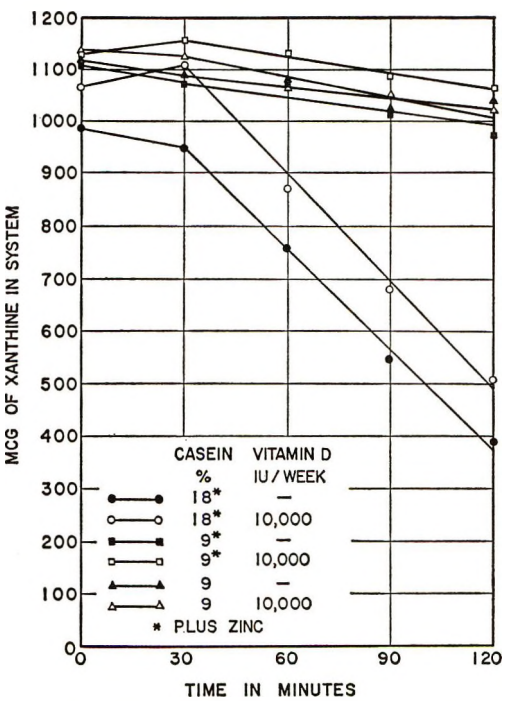

Fig. 1 Effect of protein, zinc and vitamin D on xanthine oxidase activity in liver of rats.

(fig. 1) show the highest level of residual xanthine at the end of 2 hours in liver of animals fed 9% casein plus zinc and vitamin D. The difference in the amount of xanthine in the system at zero time and 120 minutes, however, was smallest for animals fed 9% casein without supplementation and, therefore, in terms of xanthine oxidized per hour the activity per gram of liver was less for these animals. Comparison of the data recorded for the two experimental groups receiving diets containing 9% casein without vitamin D showed that xanthine oxidase activity decreased significantly when zinc was omitted from the diet ($P < 0.05$). Omission of dietary zinc had no significant effect on xanthine oxidase activity when vitamin D supplement was added to the low protein diet (table 3). The apparent interaction between zinc and vitamin D closely approached significance at the 5% level.

DISCUSSION

The concentration of seven nonessential and two essential amino acids in plasma of rats were not affected by reduction of the level of dietary protein from 18 to 9%, by supplementation of the diet providing 9% protein with zinc, or by addition of calciferol to either level of protein. The reduction of plasma methionine and phenylalanine, and the increase in level of histidine which resulted from decreased level of dietary protein, were the only sig-

TABLE 3

Xanthine oxidase activity in liver of rats fed the control diets and diets containing 9% casein with and without added zinc and vitamin D

Vitamin D	Xanthine disappearance [1]		
	18% casein + zinc	9% casein + zinc	9% casein − zinc
IU/week			
0	9.39 ± 0.44 [2]	2.06 ± 0.22	1.36 ± 0.14
10,000	9.23 ± 0.76	1.66 ± 0.21	1.74 ± 0.24

[1] Micromoles of xanthine oxidized per hour per gram of liver (moist weight).
[2] Mean of 12 animals ± SEM.

nificant changes observed for these amino acids. Plasma threonine, isoleucine, and leucine decreased when the lower level of dietary protein was fed and when diets containing added zinc were supplemented with calciferol. Lysine and alanine in plasma decreased when zinc was added to the diet which provided 9% casein. The lower concentration of lysine, which was observed when vitamin D was added to the diet, confirms the previous observation (1) that the level of plasma lysine may be related to dietary calciferol. Expressed as micromoles per 100 ml, the previously observed levels of lysine in plasma of rats fed 18% casein without added zinc, supplemented with zero and 10,000 IU of vitamin D, were 52.6 and 48.2 (1), compared with 49.1 and 39.8 recorded in the present study for corresponding animals fed a dietary source of zinc. The highest concentration of plasma lysine was recorded for animals fed the diet providing 9% casein without zinc and calciferol supplementation, which also produced the lowest activity of xanthine oxidase.

A large amount of work has been done in various studies to define the significance of alterations in plasma amino acids. Emphasis has been placed on studies of the influence of dietary modifications on amino acid patterns in plasma in order to estimate nutritional value of nutrients, particularly protein. In the broadest aspect, it appears these studies have value in determining factors which influence transport and use of amino acids.

The curves for xanthine oxidase activity in liver of rats fed 18% casein in this study are similar to the curve observed by Litwack et al. (11). The values for micromoles of xanthine oxidized per hour per gram of liver for rats fed 18% casein are similar to values reported by Cox and Harris (6) for rats fed 20% casein. The reason for the low activity of the enzyme observed during the first 30 minutes of the incubation period is not known. It is possible that naturally occurring inhibiting factors may be present in tissue.

The marked sensitivity of xanthine oxidase to dietary protein observed in this study is well known (11–14) and has been suggested as a measure of protein quality. Work by Muramatsu and Ashida (15), however, showed that soy protein which may increase the requirement for dietary zinc did not support maximal activity of xanthine oxidase. The authors concluded "that other factors besides protein level and protein adequacy can influence the enzyme activity, as in the case of soybean protein."

Results of the present study indicated that zinc supplementation in the amount of 12 mg/kg had a significant effect on the activity of xanthine oxidase in liver of rats fed 9% protein only when calciferol was omitted from the diet. Kfoury et al. (16) reported that xanthine oxidase activity expressed as micromoles of xanthine converted to uric acid per minute per milligram of nitrogen in zinc-deficient animals did not differ significantly from that of pair-fed controls when the diet provided 7.4 or 14.8% protein. Cox and Harris (6) reported that 0.4% dietary zinc decreased the activity of xanthine oxidase. Since Swenerton and Hurley (17) have recently published data which indicate that the requirement of zinc for growth and development of the rat is greater than previously reported, it is possible that the addition of 12 mg zinc to the diet used in this study

was not sufficient to demonstrate an effect on enzyme activity. Because the animals used in this study were maintained in galvanized cages the dietary zinc does not represent total zinc available to the animals. The purpose of the study was to investigate the effect of vitamin D and the addition of zinc to a diet containing no added zinc on certain body constituents of rats maintained in the same environment.

The increase in body weight which resulted from administration of vitamin D to rats fed 18% casein is in agreement with many reports. Steenbock and Herting (18) made an extensive investigation of the effect of vitamin D on growth and certain characteristics of bone of rats fed diets providing varying levels of calcium and phosphorus. Addition of the vitamin to all diets except those which contained calcium and phosphorus in the ratios of 5.42, 8.34 or 28.2 improved the weight gain of the rats and the authors concluded that "the weanling rat requires vitamin D for optimum performance." In this study, the diets containing 9% casein provided by calculation 0.122% tryptophan and 10 mg niacin/kg diet, which may be marginal for available dietary niacin (19).

Data reported in this paper suggest that the low activity of xanthine oxidase and elevated plasma lysine observed in rats fed a low protein diet without added zinc or calciferol reflected the apparent effect of calciferol on the availability or utilization of lysine for enzyme synthesis. This hypothesis is supported by the work of others (4, 5) who showed that a marginal deficiency of dietary lysine markedly decreased xanthine oxidase activity.

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Effect of Dietary Protein on Hepatic Lipogenesis in the Growing Chick

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ABSTRACT The influence of dietary protein on hepatic lipogenesis in growing chicks has been investigated. Both in vitro and in vivo studies demonstrated that the incorporation of glucose-U-^{14}C, pyruvate-2-^{14}C and acetate-1-^{14}C into liver fatty acids was depressed by elevating the dietary protein level. Liver cholesterol content, however, as well as synthesis, was increased as the dietary protein increased. The data suggest that hepatic lipogenesis and cholesterogenesis are altered by dietary protein per se regardless of its quality or its growth-stimulation effect. The plasma free fatty acid level was decreased by increasing dietary protein and was positively related to the rate of hepatic lipogenesis. Incorporation of DL-lactate-2-^{14}C and pyruvate-2-^{14}C into blood glucose appeared to be increased as the dietary protein level increased. The activity of malic enzyme was positively correlated with the rate of lipogenesis. Increasing dietary protein from 15 to 35% depressed both in vitro fatty acid synthesis and malic enzyme activity by about 75%. The possible regulatory mechanisms responsible for the depression of hepatic lipogenesis in chicks fed a high protein diet are discussed. It is suggested that a limitation in the availability of cytoplasmic reducing equivalents may initiate the reduction in hepatic fatty acid synthesis of chicks fed a high protein diet.

Recent investigations have shown that lipogenesis in liver and adipose tissue may be influenced by dietary lipid and carbohydrate. Increasing the level of dietary fat has been reported to depress fatty acid synthesis in both liver (1–5) and adipose tissue (6–8) of the laboratory rat. The lipogenic effects could be attributed to either dietary component since increasing dietary fat is generally associated with a decrease in carbohydrate levels. The work of Hill et al. (3), however, suggests that dietary fat has a specific inhibitory effect on hepatic lipogenesis unrelated to a reduced carbohydrate intake.

Numerous investigations have demonstrated that circulating lipids, particularly cholesterol and triglycerides, can be influenced by diet. Thus, dietary fat and carbohydrate have been reported to alter blood cholesterol and triglyceride levels (9, 10). Also, dietary protein influences blood lipids, particularly cholesterol levels, in growing animals (11–18). Despite these observations the effects of dietary protein on lipogenesis have not been extensively studied. Increasing the level of dietary protein has been reported to depress fatty acid synthesis in liver (1,19) and adipose tissue (7, 19) of rats and in liver of chicks (20). These effects could, however, be attributed to changes in dietary carbohydrate, since protein was increased at the expense of glucose.

The mechanisms by which various dietary components influence lipid metabolism are not clear. In the rat, an elucidation of these mechanisms is complicated by the fact that lipogenesis occurs in both liver and adipose tissue. Lipogenesis in the chicken (21, 22) and pigeon (23) has recently been shown to be restricted to the liver, with adipose tissue serving primarily as a storage organ. This species would, therefore, appear to provide some advantages in studying hepatic lipogenesis and the influence of diet.

The experiments reported here were designed to study the lipogenic response of chicks to changes in dietary fat, protein and carbohydrate. The results of these investigations show that hepatic fatty acid synthesis in the chick is depressed by increasing either dietary protein or fat, whereas the rate of cholesterogenesis is enhanced.

EXPERIMENTAL

Animals and diets. Male, crossbred chicks (New Hampshire ♂ × Columbian

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♀) were used for all experiments. The initial mean body weight of chicks for each experiment is indicated in the tables of results. The chicks were divided in such a manner as to have similar initial average body weights for each treatment group. They were housed in electrically heated cages with raised wire floors. Feed and water were supplied ad libitum, and body weight and food consumption were determined at weekly intervals. The chicks were fed the experimental diets for 3 weeks. The composition of the basal diet used in experiments 1 and 2 was as follows: (in grams per 100 g of diet) salt mixture,[1] 5.31; corn oil, 3.0; vitamin mixture,[2] 0.4; choline chloride, 0.2; and glucose, to 100. Sesame meal (47.8% protein, N × 6.25) and lysine were added to the basal diet at the expense of glucose to supply 12, 15 or 18% protein as indicated in the tables of results. Because sesame meal protein is deficient in lysine this amino acid was added to vary the quality of the protein. The effective protein level was calculated according to the amount of lysine supplemented. It was assumed that completely effective protein contained 6% lysine (25). The composition of the basal diet fed in experiments 3, 4 and 5 was identical to that for experiments 1 and 2 except that the corn oil was omitted, and 4% nonnutritive fiber was added. Isolated soybean protein (assay protein C_1, 89.0% protein, N × 6.25) and fat[3] were added to the basal diet at the expense of glucose to supply different levels of protein (10 to 40%) or lipid (5 to 20%).

Glycine and DL-methionine were added at levels equivalent to 2.0 and 1.5% of the protein, respectively. As the level of dietary fat was increased the protein level was altered to maintain a constant calorie-to-protein ratio.

METHODS

At the termination of the experiments the chicks were killed by cervical dislocation and the liver was rapidly excised and weighed. A portion of the liver used for the preparation of liver slices was placed in ice-cold saline (0.9% NaCl). Liver slices, prepared with a Stadie-Riggs hand microtome, were used to study the incorporation of various ^{14}C-labeled substrates into fatty acids and cholesterol. The incubation conditions and procedures for isolating and determining the radioactivity of fatty acids and cholesterol have been described. (26).

Gluconeogenesis and lipogenesis from DL-lactate-2-^{14}C, pyruvate-2-^{14}C, glucose-U-^{14}C and acetate-1-^{14}C were determined in vivo in experiments 4 and 5 (tables 4 and 6). Each chick was given intravenously 0.5 ml of saline containing: 1 mmole and 1.2 μCi of DL-lactate-2-^{14}C (sodium salt) in experiment 4; 3.5 μCi pyruvate-2-^{14}C (sodium salt), 4.6 μCi glucose-U-^{14}C or 4.7 μCi acetate-1-^{14}C (sodium salt) in experiment 5. Thirty minutes (exp. 4) and 15 minutes (exp. 5) later blood was obtained by cardiac puncture using a heparinized syringe; then the chicks were killed and the livers quickly removed. Lipids were isolated from liver by the procedure of Folch et al. (27). The isolated lipids were saponified; the fatty acids were isolated and the radioactivity determined by the same procedure as used for the in vitro studies. The gluconeogenic capacity was estimated by determining the radioactivity of plasma glucose. The plasma was deproteinized by the Somogyi procedure (28), and the protein-free filtrate was passed over a column containing a mixture of IR-120 (H^+ form) and IR-45 (OH^- form). One milliliter of the column eluates was mixed with 10 ml of Bray's solution (29) for assay of radioactivity. Glucose was determined by using a commercial glucose oxidase reagent.[4]

In one experiment body fat was estimated from body water values. The carcass was dried to constant weight at 100° in a forced-draft oven, and the loss of weight was taken as body water. Body fat was calculated by means of an equation derived from the data of Summers and Fisher (30) for crossbred chicks of similar body weight as used in the present studies.

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[1] For composition of salt mixture see Leveille et al. (24).

[2] The vitamin mixture supplied the following (in milligrams per 100 g of diet) thiamine·HCl, 2.2; riboflavin, 2.2; Ca pantothenate, 6.6; pyridoxine·HCl, 2.2; biotin, 0.06; folic acid, 0.4; *p*-aminobenzoic acid, 11; menadione, 5; vitamin B_{12}, 0.03; inositol, 10; ascorbic acid, 20; niacin, 10; and α-tocopheryl acetate, 10; and (in IU) vitamin A acetate, 2000; and vitamin D_3, 220.

[3] Crisco, Procter and Gamble, Cincinnati, Ohio.

[4] Glucostat, Worthington Biochemical Corporation, Freehold, N. J.

The equation was $y = 71.785 - 0.920\ x$, where y = percentage body fat and x = percentage body water.

Total liver fat and cholesterol were determined as previously described (31). Liver glycogen was isolated by co-precipitation with Na_2SO_4 (32) following the digestion of liver tissue in hot 30% KOH. The precipitated glycogen was quantitated by means of the anthrone reaction (33). Plasma free fatty acids were determined by the methods of Ko and Roger (34). Liver was homogenized in 0.15 M KCl and centrifuged at 100,000 × *g* for 1 hour. Activity of malic enzyme (EC. 1.1.1.40) in the supernatant fraction was assayed by the method of Ochoa (35). The protein content of the supernatant used for enzyme assay was determined by the method of Lowry et al. (36). Enzyme activities are expressed as units per milligram protein where a unit is the amount of enzyme which will catalyze the formation of 1 mμmole NADPH/minute at 30°.

Statistical analysis. The data were analyzed statistically by means of the analysis of variance.

RESULTS

Experiment 1. The effect of level and quality of dietary protein on hepatic lipogenesis is shown in table 1. Diets 1, 2 and 3 were formulated to contain different levels of protein (12, 15 and 18%) of the same quality (adequately supplemented with lysine), whereas, diets 4, 5 and 6 contained 18% sesame protein with different levels of lysine, and consequently, of effective protein. The growth of chicks increased as the level of effective protein increased from 8 to 18% of the diet. Feed efficiency paralleled the weight gain, being improved by higher levels of effective protein. Liver slices from chicks fed the highest protein-containing diet incorporated significantly less acetate-1-^{14}C into fatty acids than comparable preparations from birds consuming the lower levels of protein. Body fat followed the same trend as fatty acid synthesis, being depressed by increasing protein levels. Similar rates of fatty acid synthesis and body fat were observed in animals of groups 4 and 6 which were fed diets containing 18% total protein, but with different levels of effective

TABLE 1

Influence of dietary protein level on weight gain, feed efficiency, body and liver lipids and in vitro hepatic lipogenesis of growing chicks [1]

	Treatment group [2]						P [3]	
	1	2	3	4	5	6	Groups 1–3	Groups 4–6
Dietary protein, %	12	15	18	18	18	18	—	—
Dietary lysine, % [4]	0.72	0.90	1.07	0.47	0.72	1.07	—	—
Effective protein, %	12	15	18	8	12	18	—	—
Body wt gain, g	400 ± 9 [5]	488 ± 11	538 ± 16	160 ± 19	387 ± 17	529 ± 12	< 0.01	< 0.01
Gain, grams per gram feed consumed	0.42	0.50	0.57	0.29	0.45	0.52	—	—
Liver fat, %	4.8 ± 0.1	4.6 ± 0.2	5.4 ± 0.2	4.8 ± 0.2	4.7 ± 0.1	4.8 ± 0.1	ns	ns
Liver cholesterol, mg/g	2.8 ± 0.1	2.9 ± 0.1	3.8 ± 0.3	3.2 ± 0.1	3.1 ± 0.1	3.7 ± 0.3	< 0.01	< 0.01
Body fat, %	14.9 ± 0.4	13.2 ± 0.4	10.3 ± 0.2	10.8 ± 1.0	12.7 ± 0.5	10.3 ± 0.2	< 0.01	< 0.01
Fatty acid synthesis [6]	1474 ± 101	838 ± 63	565 ± 72	652 ± 46	1067 ± 73	668 ± 88	< 0.01	< 0.05

[1] Initial body weight = 145 ± 2 g.
[2] Groups 3 and 6 are replicates.
[3] Probability of a significant treatment effect; ns = not significant.
[4] The figures shown represent the sum of the estimated lysine content of sesame meal plus supplemental lysine.
[5] Mean for five chicks ± SEM.
[6] Millimicromoles acetate-1-^{14}C incorporated into fatty acids per 100 mg liver/2 hours.

protein (8 versus 18%). A higher rate of fatty acid synthesis and body fat, however, were observed in chicks of group 5 which were fed a diet supplying 12% effective protein. Total liver lipids and cholesterol were not markedly altered by the diets fed although the treatment effect on liver cholesterol level was statistically significant. The results of this experiment suggest that hepatic lipogenesis was altered by the level of dietary protein regardless of its quality; however, the high rate of fatty acid synthesis observed for treatment group 5 makes such a conclusion equivocal.

Experiment 2. If lipogenesis was influenced by the level of dietary protein per se, rather than by an effect of protein on growth, the rate of lipogenesis of groups 4 through 6 in experiment 1 should have been similar. In these groups the total dietary protein level was constant but the growth-promoting effect of the protein was altered by lysine supplementation. The data from groups 4 and 6 demonstrate that despite a marked growth difference the rate of lipogenesis and level of body fat were similar. Group 5, which grew at a rate intermediate to the chicks in groups 4 and 6, however, had a higher rate of hepatic lipogenesis and a greater amount of body fat. Consequently, it was not possible to arrive at a conclusion concerning the relationship between dietary protein, growth and hepatic lipogenesis. This portion of the experiment (groups 4, 5 and 6) therefore was repeated. As shown in table 2, the results of this experiment were in general agreement with those of experiment 1 except that the rate of fatty acid synthesis was similar for all groups despite the fact that body weight gain and feed efficiency were improved by increasing the level of effective protein. This demonstrated that protein quality does not influence hepatic lipogenesis in the chick.

It should be noted that the rate of acetate-1-^{14}C incorporation into fatty acids by liver slices was higher in experiment 2 than for similar groups in experiment 1. Such variations in absolute rates of fatty acid synthesis between experiments are not uncommon and consequently invalidate comparisons between experiments.

Experiment 3. This experiment was designed to compare the influence of dietary protein and fat on hepatic lipogenesis. The observed results are summarized in table 3 and figure 1. Increasing dietary protein or fat, and consequently decreasing the calories derived from carbohydrate, depressed hepatic lipogenesis. The slopes of the regression lines shown in figure 1 were significantly different ($P < 0.025$), implying that the effect of protein is greater than that of fat, and suggesting that dietary fat and protein depress fatty acid synthesis through different mechanisms. Feed efficiency was improved by increasing the dietary protein level as observed in experiments 1 and 2.

Experiment 4. The depression in hepatic lipogenesis induced by increasing protein levels might be due to decreased dietary glucose rather than increased protein. Nevertheless, high levels of dietary protein could directly influence some metabolic pathways (e.g., reversal of glycolysis) which, in turn, could affect lipogenesis. Experiment 4 was carried out to determine whether the protein level influenced the rate of gluconeogenesis in the chick (table 4).

TABLE 2

Effect of lysine supplementation of diets containing 18% sesame protein on weight gain, feed efficiency and in vitro hepatic lipogenesis of growing chicks [1]

	Treatment group			P [2]
	4	5	6	
Effective protein, %	8	12	18	—
Body wt gain, g	72 ± 6 [3]	280 ± 14	463 ± 11	< 0.01
Gain, grams per gram feed consumed	0.28	0.46	0.57	—
Fatty acid synthesis [4]	1690 ± 182	1373 ± 115	1374 ± 250	ns

[1] Initial body weight = 96 ± 6 g. Treatments are identical to groups 4, 5 and 6 in table 1.
[2] Probability of a significant treatment effect; ns = not significant.
[3] Mean for five chicks ± SEM.
[4] Millimicromoles acetate-1-^{14}C incorporated into fatty acids per 100 mg liver/2 hours.

TABLE 3

Effect of dietary protein and fat on in vitro hepatic lipogenesis, weight gain, feed efficiency and liver lipid levels of growing chicks [1]

	Treatment group							P [2]	
	1	2	3	4	5	6	7	Groups 1–4	Groups 2, 5–7
Dietary protein, %	10	20	30	40	21	22	23	—	—
Dietary fat, %	5	5	5	5	10	15	20	—	—
Carbohydrate calories, %	78	68	56	44	56	47	39	—	—
Weight gain, g	221 ± 29 [3]	483 ± 11	519 ± 19	518 ± 22	510 ± 17	432 ± 17	447 ± 21	< 0.01	< 0.05
Gain, grams per gram feed consumed	0.36	0.58	0.67	0.69	0.64	0.65	0.68	—	—
Fatty acid synthesis [4]	2247 ± 64	1478 ± 74	1115 ± 71	403 ± 36	1263 ± 156	1096 ± 88	878 ± 87	< 0.01	< 0.05

[1] Initial body weight = 93 ± 2 g.
[2] Probability of a significant treatment effect.
[3] Mean for five chicks ± SEM.
[4] Millimicromoles acetate-1-^{14}C incorporated into fatty acids per 100 mg liver/2 hours.

There was a suggestion of an increased incorporation of DL-lactate-2-^{14}C into blood glucose as the dietary protein level increased. Liver glycogen was not consistently influenced by dietary protein. Hepatic lipogenesis, determined in vitro and in vivo, was depressed by increasing dietary protein levels as were plasma free fatty acids. Cholesterol synthesis in vitro increased significantly as the protein level was increased.

Experiment 5. Various substrates, namely glucose-U-^{14}C, pyruvate-2-^{14}C and acetate-1-^{14}C were used in this experiment to investigate the rate of hepatic lipogenesis in vitro and in vivo. In addition, gluconeogenesis from pyruvate-2-^{14}C was studied. The results of in vitro and in vivo studies (tables 5 and 6) demonstrated that the rate of incorporation of glucose-U-^{14}C, pyruvate-2-^{14}C and acetate-1-^{14}C into liver fatty acids was depressed by increasing the dietary protein level, whereas glucose production from pyruvate-2-^{14}C appeared to be increased (table 6). The activity of malic enzyme (table 6) was positively correlated with the rate of lipogenesis (tables 5 and 6). As can be seen, increasing the level of dietary protein from 15 to 35% decreased the hepatic malic enzyme activity by about 75%.

DISCUSSION

Although hepatic lipogenesis has been extensively studied in the rat it is often difficult to interpret the physiological significance of these results because the liver synthesizes a relatively small portion of total fatty acids (37, 38). Futhermore, lipogenesis in liver and extrahepatic sites may respond quite differently to a particular treatment (39). The domestic chick was used for the present studies because liver is the major site of fatty acid synthesis in this species (21, 22) and overall lipogenesis is, therefore, reflected by hepatic lipid synthesis.

Several reports have demonstrated that in rats and mice hepatic and adipose tissue lipogenesis was reduced by increasing the level of dietary fat (2–4). The chick also appeared to respond to increased ingestion of fat with a reduced rate of hepatic fatty acid synthesis (40), although the results of this study were equivocal since the incubation conditions employed could not

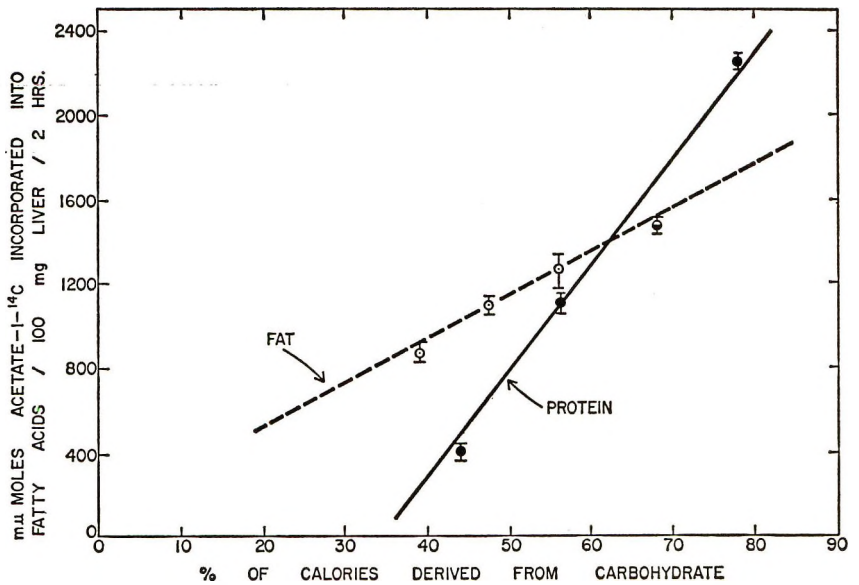

Fig. 1 Effect of dietary protein and fat level on hepatic lipogenesis in growing chicks. Each point represents the mean value for five chicks ± SEM. The point represented by the half-filled circle is common to both lines. Dietary levels of protein and fat, and the percentage of calories derived from carbohydrate are as shown in table 3. The two regression lines are significantly different from each other ($P < 0.025$).

TABLE 4

Effect of dietary protein on gluconeogenesis and lipogenesis in growing chicks [1]

	Treatment group			P [2]
	1	2	3	
Dietary protein, %	20	30	40	—
Body wt gain, g (10)	363 ± 16 [3]	418 ± 6	418 ± 5	< 0.01
Fatty acid synthesis [4] (5)	1585 ± 124	1279 ± 243	224 ± 96	< 0.01
Cholesterol synthesis [4] (5)	20 ± 4.2	38 ± 6.2	64 ± 9.5	< 0.01
Plasma FFA, μEq/liter (10)	534 ± 55	419 ± 34	320 ± 28	< 0.01
Liver glycogen, mg/g (5)	24 ± 4.0	30 ± 4.4	14 ± 2.7	< 0.05
DL-Lactate-2-^{14}C converted to: [5]				
Blood glucose, 10^3 dpm (5)	997 ± 95	1098 ± 56	1241 ± 15	ns
Liver fatty acids, dpm/100 mg liver (5)	262 ± 118	45 ± 11	20 ± 8	ns

[1] Initial body weight = 102 ± 1 g. All diets contained 5% fat.
[2] Probability of a significant treatment effect; ns = not significant.
[3] Mean ± SEM for number of chicks shown in parentheses.
[4] Millimicromoles acetate-1-^{14}C incorporated into fatty acids or cholesterol per 100 mg liver/2 hours.
[5] Each chick was given intravenously 0.5 ml of saline containing 1 mmole and 1.2 μCi of sodium DL-lactate-2-^{14}C and killed after 30 minutes. Total glucose radioactivity was estimated by assuming a glucose space equivalent to 42% of body weight (65).

yield quantitative information because of substrate limitation (41). The present investigations clearly showed that increasing dietary fat levels depressed lipogenesis in the chick.

Although the effect of dietary protein on fatty acid synthesis has not been extensively investigated it has been reported that increasing the dietary protein level decreased fatty acid synthesis in liver and

TABLE 5

Influence of dietary protein level on weight gain, feed efficiency and in vitro hepatic lipogenesis of growing chicks [1]

	Treatment group			P [2]
	1	2	3	
Dietary protein, %	15	25	35	—
Body wt gain, g	373 ± 15 [3]	469 ± 22	510 ± 36	< 0.01
Gain, grams per gram feed consumed	0.41	0.53	0.56	—
Fatty acid synthesis from: [4]				
Glucose-U-^{14}C	821 ± 63	281 ± 80	110 ± 39	< 0.01
Pyruvate-2-^{14}C	1461 ± 234	562 ± 88	445 ± 92	< 0.01
Acetate-1-^{14}C	3278 ± 239	1732 ± 335	982 ± 163	< 0.01

[1] Initial body weight = 109 ± 2 g. All diets contained 5% fat.
[2] Probability of a significant treatment effect.
[3] Mean for five chicks ± SEM.
[4] Millimicromoles labeled substrate incorporated into fatty acids per 100 ml liver/2 hours.

TABLE 6

Influence of dietary protein level on malic enzyme activity, glucose synthesis and in vivo hepatic lipogenesis in growing chicks [1]

	Treatment group			P [2]
	1	2	3	
Dietary protein, %	15	25	35	—
NADP-malate dehydrogenase [3]	107 ± 8 [4]	50 ± 4	26 ± 2	< 0.01
Pyruvate-2-^{14}C converted to blood glucose, 10^3 dpm [5]	1376 ± 162	1738 ± 117	1973 ± 279	ns
Fatty acid synthesis from: [5]				
Pyruvate-2-^{14}C, % of dose	3.4 ± 1.9	0.6 ± 0.2	0.2 ± 0.1	< 0.01
Acetate-1-^{14}C, % of dose	9.1 ± 0.9	2.7 ± 0.5	2.7 ± 0.8	< 0.01

[1] Initial body weight 109 ± 2 g. All diets contained 5% fat.
[2] Probability of a significant treatment effect; ns = not significant.
[3] Values are millimicromoles NADPH formed per minute per milligram protein.
[4] Mean for five chicks ± SEM.
[5] Chicks were given intravenously in 0.5 ml saline, 4.7 μCi acetate-1-^{14}C or 3.4 μCi pyruvate-2-^{14}C. Fifteen minutes later the chicks were killed and the livers were removed. Total glucose radioactivity was estimated by assuming a glucose space equivalent to 42% of body weight (65). Fatty acid synthesis represents percentage of the administered dose recovered in liver fatty acids.

adipose tissue of rats (7, 19) and in liver of chicks (20). The data presented in this report are in accord with these findings. The influence of dietary protein on circulating cholesterol levels in chicks has received considerable attention. It has been shown that serum cholesterol levels are inversely related to dietary protein level in this species (11–17); similar observations have been reported for growing swine (18). Nishida et al. (20) attributed the cholesterol-depressing effect of a high protein diet to its ability to depress hepatic cholesterogenesis or to increase cholesterol catabolism, or both (16). The results presented in table 1 suggest that liver cholesterol may be elevated by increased dietary protein levels, and the data in table 4 demonstrate an increased capacity for cholesterogenesis by liver of chicks fed increasing levels of dietary protein. The elevated level of cholesterol in the liver of chicks fed the higher protein-containing diets (table 1) was in accord with a previous report (42). These observations would suggest that the blood cholesterol lowering effect of protein is due to its influence on cholesterol catabolism rather than on synthesis.

The mechanisms by which dietary fat, and particularly dietary protein, depress hepatic lipogenesis are not clear. The path-

way of fatty acid biosynthesis has been elucidated (43) and is known to involve the following sequence of reactions:

$$(1)\ \text{Acetyl-CoA} + CO_2 + \text{ATP} \xrightarrow[\text{carboxylase}]{\text{Acetyl-CoA}} \text{malonyl CoA} + \text{ADP} + \text{Pi}$$

$$(2)\ \text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\text{NADPH} \xrightarrow[\text{synthetase complex}]{\text{Fatty acid}} \text{palmitate} + 14\text{NADP}^+ + 8\text{CoA} + 7CO_2 + 6H_2O$$

The active intermediates in reaction 2 are presumed to be acyl carrier protein derivatives rather than coenzyme A derivatives (44). It is generally agreed that the acetyl-CoA carboxylase enzyme catalyzing reaction 1 is the rate-limiting step, not necessarily because of a limiting amount of enzyme as had been previously thought (45, 46), but because of the allosteric nature of this enzyme (47-49). It is known that the activity of the enzymes catalyzing both reactions 1 and 2 is adaptive; however, it is unlikely that changes in enzyme activity per se are responsible for the initial alteration in lipogenic activity. In accord with this is the observation of Masoro (50) that fasting in the rat depressed hepatic fatty acid synthesis to a greater extent than it does the activity of acetyl-CoA carboxylase or fatty acid synthetase. Furthermore, studies of the activity of other enzymes related to fatty acid synthesis such as pentose pathway dehydrogenases, malic enzyme and citrate cleavage enzyme show that changes in their activity are preceded by a change in the rate of fatty acid synthesis (26, 51). Such observations suggest that the lipogenic enzymes adapt to a change in the rate of fatty acid synthesis, and consequently, that alterations in the rate of fatty acid synthesis are not directed by alteration in enzyme activity. If this is the case, then one must look to other factors in attempting to ascertain the mechanism involved in depressing lipogenesis when carbohydrate intake is reduced by increased levels of dietary fat or protein.

Long-chain fatty acids have been reported to depress fatty acid synthesis, presumably by inhibiting acetyl-CoA carboxylase and fatty acid synthetase activities (52, 53). Although there is some question as to whether long-chain fatty acyl-CoA derivatives act directly (54) it appears clear that they do somehow limit fatty acid synthesis. Such a mechanism could be invoked to explain the depressing effect of dietary fat on hepatic lipogenesis. The mechanism involved in the protein effect, however, appears more complex. That long-chain fatty acids are not involved is suggested by the data in table 4 which show that as dietary protein increased both the rate of fatty acid synthesis and the level of circulating free fatty acids were depressed. Other factors which conceivably might limit fatty acid synthesis are substrate availability and the rate of generation of reducing equivalents to support fatty acid biosynthesis. It seems unlikely that the production of acetyl-CoA would be limiting since the highest levels of protein fed still allowed for a substantial amount of carbohydrate in the diet, and the chick can readily catabolize the amino acids derived from dietary protein.

The chick differs from the rat in terms of the pathways available for the production of NADPH. The pentose pathway apparently is not active in chick liver, at least not active enough to supply any appreciable amount of NADPH (21, 55–58). Also, NADP-dependent isocitrate dehydrogenase, another potential source of cytoplasmic reducing equivalents, does not appear to be important in this regard for the support of lipogenesis in chick liver (57). In the rat the pentose pathway, although active, can supply only approximately 50% of the reducing equivalents needed for high rates of fatty acid synthesis (59). It has been proposed that the remainder could be supplied by a transhydrogenation sequence involving NAD-malic dehydrogenase and malic enzyme in which reducing equivalents are transferred from NADH to $NADP^+$ (60–62). Ballard and Hanson (63) have elaborated this scheme by demonstrating that in the presence of cytoplasmic pyruvate carboxylase a "transhydrogenase cycle" is formed which has the potential of producing large quantities of NADPH.

In an earlier report (21) from this laboratory it was proposed that in view of the activity of malic enzyme in chick liver this cycle is probably the major source of reducing equivalent for the support of fatty

acid biosynthesis in the chick. Goodridge and Ball (64) arrived at a similar conclusion in their studies with the pigeon. This suggestion is supported by the adaptive nature of malic enzyme in chick liver (58 and present study). The functioning of the transhydrogenase cycle is dependent upon the availability of cytoplasmic NADH normally generated in the glycolytic pathway. If protein is serving as a major source of acetyl-CoA the production of cytoplasmic NADH could be limited, and, in turn, the generation of NADPH might be reduced, thereby limiting fatty acid synthesis. Furthermore, if glucose production is enhanced when dietary protein is increased, as suggested by the data in tables 4 and 6, this would utilize cytoplasmic NADH and further reduce that available for transhydrogenation. We would, therefore, suggest that the availability of cytoplasmic-reducing equivalents is probably the major factor limiting fatty acid synthesis in the chick fed high levels of dietary protein. In support of this proposal is the close relationship noted between the in vitro capacity for fatty acid synthesis and malic enzyme activity. Increasing dietary protein from 15 to 35% depressed both in vitro fatty acid synthesis (table 5) and malic enzyme activity (table 6) by about 75%. Studies are presently in progress to substantiate this postulation.

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In vivo Fatty Acid and Cholesterol Synthesis in Fasted and Fasted-refed Chicks

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ABSTRACT The effects of fasting and refeeding following a fast on in vivo hepatic fatty acid and cholesterol synthesis were studied in growing chicks. Also investigated was the influence of these treatments on liver lipid and glycogen content and on the activities of hepatic glucose 6-phosphate and 6-phosphogluconate dehydrogenases and malic enzyme. Hepatic fatty acid and cholesterol synthesis were significantly depressed by a 24-hour fast and continued to decrease during the 3-day starvation period studied. Fatty acid synthesis increased to 275% and 364% of normal after 1 and 2 days of refeeding, respectively, following a 3-day fast. On day 3 of refeeding, fatty acid synthesis returned to normal and remained so throughout the remainder of the 5-day refeeding period. Cholesterol synthesis remained depressed until day 3 of refeeding, when a significant increase was observed, and reached near-normal values on day 4. The activity of the pentose pathway dehydrogenases was very low in chick liver and was not greatly influenced by fasting or refeeding. Malic enzyme activity was severely decreased by fasting and returned to control values after 2 days of refeeding. Liver weight and liver glycogen decreased markedly during the fasting period, increased to above normal values during the first 2 days of refeeding and returned to control levels after 3 days of refeeding. Liver total lipid was not significantly influenced by fasting but increased upon refeeding and began to decrease after 3 days of refeeding. Liver cholesterol content increased during fasting and decreased rapidly upon refeeding.

The consequences of fasting and refeeding following a fast on lipid metabolism in the laboratory rat have been extensively investigated and the results of these studies have been reviewed (1–4). In the rat, fasting markedly depresses hepatic lipogenesis, and refeeding following a fast induces a marked increase in fatty acid synthesis. Accompanying these changes in fatty acid biosynthesis are parallel changes in the activity of several enzymes related to fatty acid synthesis. Thus, the activity of hepatic pentose pathway dehydrogenases, malic enzyme, citrate cleavage enzyme, glucokinase, acetyl-CoA carboxylase and fatty acid synthetase decrease with fasting and increase to above normal levels upon refeeding following a fast.

The effects of fasting and refeeding have not, however, been extensively studied in avian species. The study of hepatic lipogenesis and its control in these species is of particular interest since lipid synthesis in birds is largely restricted to the liver (5–8). Also, other aspects of hepatic lipid metabolism appear to be different in birds than in mammals. For example, it has been well documented that fasting increases liver fat in the rat (9) as well as in many other species (as cited in 10), yet fasting chickens do not develop a fatty liver (10, 11). The consequence of fasting and refeeding following a fast on hepatic fatty acid and cholesterol synthesis in the chicken has only recently been studied. Hepatic fatty acid and cholesterol synthesis, measured in vitro, have been shown to be depressed by fasting and markedly stimulated by refeeding following a fast (12). The effect of fasting and refeeding on lipid synthesis in intact chicks has not been investigated, however. The value of in vivo investigations in establishing the physiological significance of in vitro observations has been considered in detail by Favarger (13).

The studies reported here were designed to investigate the effects of fasting and refeeding following a fast on in vivo lipogenesis in the chick. In addition, the effects of these treatments on liver weight and on liver content of total lipid, cholesterol and glycogen were determined.

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EXPERIMENTAL

Male crossbred chicks (Columbian ♀ × New Hampshire ♂) weighing about 500 g were used. The chicks were fed ad libitum until the time of the experiments and water was available at all times. The periods of fasting and refeeding are indicated in the Results section. A practical corn-soybean oil meal diet was fed which supplied 23% crude protein (N × 6.25) and 4.5% fat. The diet supplied about 61, 27 and 12% of the calories as carbohydrate, protein and fat, respectively.

Lipogenesis in vivo was studied by administering 0.5 ml of 0.9% NaCl containing 4.4 μCi of sodium acetate-1-^{14}C (specific activity 2 μCi/μmole) into a leg vein. Fifteen minutes after administering the radioactive tracer the chicks were killed by cervical dislocation; the liver was rapidly removed and chilled on ice. It has been shown that at this time over 90% of synthesized fatty acids are in the liver (8). A weighed piece of liver (100 to 200 mg) was saponified in 10 ml of 10% KOH in 95% methanol for 3 hours. Following the addition of 10 ml of water the nonsaponifiable lipids were extracted with three 5-ml portions of petroleum ether (bp 50 to 60°). The ether extracts were combined, and cholesterol was isolated and counted as the digitonide (14). The aqueous fraction containing the saponified lipids was acidified and the fatty acids were extracted with three 5-ml portions of petroleum ether. The petroleum ether extracts were combined in a liquid scintillation vial; the ether was evaporated under a stream of N_2 and the fatty acid residue was dissolved in 10 ml of scintillant solution (4 g Omnifluor, 230 ml ethanol and toluene to 1 liter). Radioactivity was determined in a liquid scintillation spectrometer.[1]

At the time of killing, a weighed piece of liver (100 to 200 mg) was rapidly removed and was digested in hot 30% KOH for the isolation and quantitation of glycogen as previously described (15). Liver total lipids and cholesterol were determined by procedures outlined previously (16, 17). A portion of the liver was also taken for enzyme assay. The tissue was homogenized with a 0.15 M KCl solution; the homogenate was centrifuged at 100,000 × *g* for 1 hour and the resulting supernatant was used for enzyme assay. Glucose 6-phosphate dehydrogenase (EC.1.1.1.49) and 6-phosphogluconate dehydrogenase (EC.1.1.1.44) were assayed by the method of Glock and McLean (18). Malic enzyme (EC.1.1.1.40) was assayed by the method of Ochoa (19). The protein content of the tissue homogenate used for enzyme assay was determined by the procedure of Lowry et al. (20).

RESULTS

A preliminary experiment was conducted to study the effect of fasting and refeeding following a fast on liver glycogen and lipid content, and on the activity of dehydrogenases in liver. The results of this experiment are presented in table 1. As shown in table 1, liver weight relative to body weight decreased by about 30% as a consequence of fasting (3 days), and during the same period the glycogen content of liver decreased by over 90%. Refeeding for 3 days following a 3-day fast returned liver weight and glycogen content to normal. Liver fat was not influenced by fasting but increased significantly ($P < 0.05$) upon refeeding (table 1). Liver cholesterol increased by over 60% as a result of a 3-day fast and decreased upon refeeding. Malic enzyme activity decreased by over 75% during a 3-day fast, and refeeding for 3 days returned activity to near normal. The activity of the pentose pathway enzymes, glucose 6-phosphate and 6-phosphogluconate dehydrogenases was low and not markedly altered by fasting and refeeding. The activity of 6-phosphogluconate dehydrogenase, however, was significantly lower ($P < 0.01$) in livers of fasted chicks.

The effect of fasting and refeeding was studied more extensively in the experiment summarized in figures 1 through 5. The daily changes in various liver constituents and in vivo lipogenesis resulting from fasting for 3 days and refeeding for 5 days (following a 3-day fast) were determined. The relative changes in body weight induced by fasting and refeeding are shown

[1] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

TABLE 1

Influence of fasting and refeeding following a fast on liver weight, on liver glycogen and lipid content and on liver enzymes of chicks [1]

	Treatment		
	Fed	Fasted	Fasted-refed
Liver: [2]			
Weight, g/100 g body wt	2.87 ± 0.10 [3]	1.98 ± 0.05	3.36 ± 0.14
Glycogen, mg/g	28.7 ± 2.0	1.9 ± 0.7	27.6 ± 6.3
Total lipids, mg/g	54.7 ± 2.3	52.9 ± 1.5	71.9 ± 6.1
Total cholesterol, mg/g	4.8 ± 0.2	6.9 ± 0.3	5.9 ± 0.2
Liver enzyme: [4]			
Malic enzyme	102 ± 5	23 ± 1	79 ± 4
Glucose 6-phosphate dehydrogenase	1.6 ± 0.2	1.5 ± 0.1	2.3 ± 0.8
6-Phosphogluconate dehydrogenase	6.4 ± 0.2	4.7 ± 0.3	6.6 ± 0.3

[1] Chicks weighing 485 ± 7 g were used. Fed chicks had access to food until the time of killing; fasted chicks were starved for 3 days; fasted-refed chicks were starved for 3 days then fed ad libitum for 3 days prior to killing.
[2] All values are expressed on a wet weight basis.
[3] Mean for five chicks ± SE.
[4] Enzyme values are expressed as nanomoles of substrate utilized per milligram per minute at 25°. Each value represents the mean for five chicks ± SE.

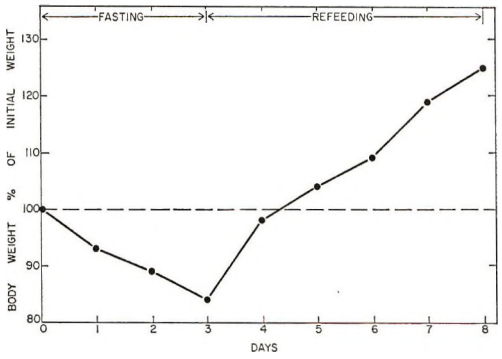

Fig. 1 Effect of fasting and refeeding on body weight of chicks. Values are expressed as a percentage of initial body weight. Initial body weight was 523 ± 8 g. Each point represents the mean for five chicks.

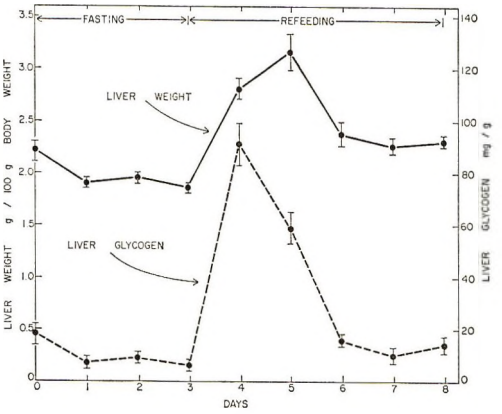

Fig. 2 Weight and glycogen content of livers from fasted and fasted-refed chicks. Values are expressed on a wet weight basis. Each point represents the mean ± SEM for five chicks.

in figure 1. As expected, the chicks lost weight during the fast and gained weight as a consequence of refeeding. Liver weight, however, decreased more rapidly than total body weight during the period of fast and increased more rapidly during the first 2 days of refeeding (fig. 2). After 3 days of refeeding, liver weight relative to body weight was similar to the value for normal chicks fed ad libitum and remained so through day 5 of refeeding. Liver glycogen content followed essentially the same pattern as that for liver weight (fig. 2) except that a peak was observed after 1 day of refeeding. Liver total lipids did not change significantly during the fasting period (fig. 3) although there was a suggestion of an increase; this amounted to a change of only 10%. Upon refeeding, liver lipids increased to a maximum after 2 days and then decreased. Liver cholesterol increased gradually during the period of fast, appeared to decrease following 1 day of refeeding and to increase slightly thereafter (fig. 3).

The effects of fasting and refeeding on in vivo lipogenesis and cholesterogenesis are shown in figure 4. Both fatty acid and

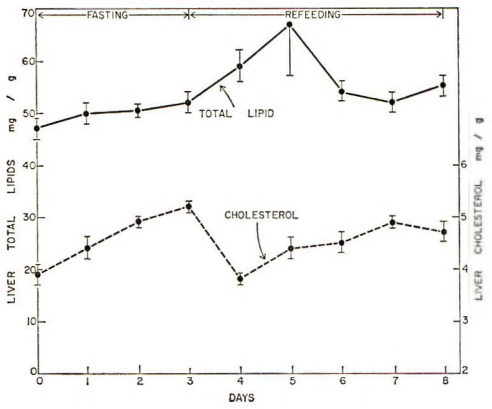

Fig. 3 Lipid and cholesterol levels in livers from fasted and fasted-refed chicks. Values are expressed on a wet weight basis. Each point represents the mean ± SEM for five chicks.

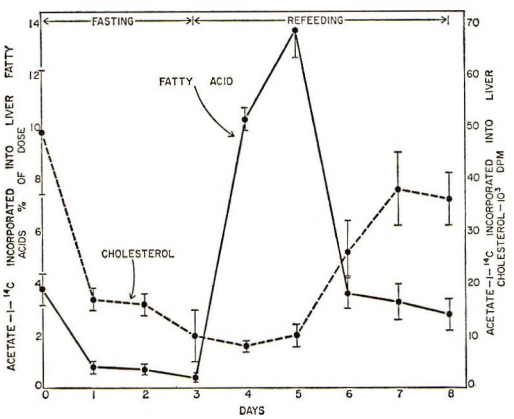

Fig. 4 In vivo hepatic fatty acid and cholesterol synthesis in fasted and fasted-refed chicks. Each point represents the mean ± SEM for five chicks.

cholesterol synthesis followed essentially the same pattern during the period of fast, decreasing markedly on day 1 and continuing to decrease more slowly thereafter. Fatty acid synthesis was depressed by about 90% and cholesterol synthesis by approximately 80% as a result of the 3-day fast. The responses of hepatic fatty acid and cholesterol synthesis to refeeding were quite different. Fatty acid synthesis increased rapidly upon refeeding, reached a peak after 2 days of refeeding and then subsided on day 3 to normal rates of fatty acid synthesis. The peak values for fatty acid synthesis reached after 1 and 2 days of refeeding were three- to fourfold greater than the rate of synthesis noted in control chicks. Cholesterol synthesis remained at about 20% of control values during the first 2 days of refeeding, increased markedly to about 50% of control values on day 3, and to about 75% of control after 4 and 5 days (fig. 4). It is obvious from the data shown in figure 4 that during refeeding cholesterol synthesis remained suppressed until fatty acid synthesis had returned to the control value and then it began to increase.

The observed changes in malic enzyme activity are shown in figure 5. The activity dropped rapidly following day 1 of the fast, and more gradually thereafter to an activity equivalent to about 30% of control values. The activity increased rapidly to control levels upon refeeding. It is of significance that there was little "overshoot" upon refeeding but rather a return to a near-normal value.

DISCUSSION

In vitro measurement of hepatic fatty acid or cholesterol synthesis generally represents the maximal capacity of the tissue, and hence, reflects the enzymic potential for such synthesis. The existence of an enzymic potential, however, does not necessarily imply that this level of activity will be observed in vivo. In the intact animal the enzymic potential is generally

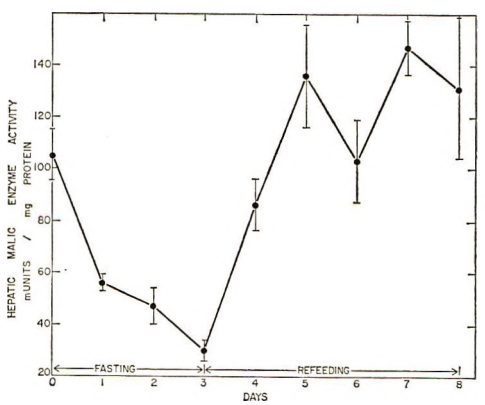

Fig. 5 Hepatic malic enzyme activity in fasted and fasted-refed chicks. Values are expressed as milliunits per milligram protein and a unit is defined as the transformation of 1 micromole of substrate per minute at 25°. Each point represents the mean ± SEM for five chicks.

modulated and often severely suppressed. Consequently, both in vitro and in vivo studies are necessary to gain an understanding of the regulation of lipid synthesis in the intact organism. These concepts have been discussed in detail by Favarger (13) who also presented data illustrating the importance of correlating in vitro and in vivo experiments.

A comparison of the results obtained in the present study and those reported by Goodridge (12) demonstrates the value of correlating in vitro and in vivo observations. The results of both the investigations are in general agreement and illustrate that in the chick, as in the rat (1–4), fasting depresses hepatic fatty acid and cholesterol synthesis, and refeeding following a fast markedly stimulates hepatic synthesis of these lipids. Yet a closer examination of the results of these two investigations illustrates some differences of physiological significance. Fasting for 3 days virtually abolished hepatic fatty acid and cholesterol synthesis measured in vitro (12) or in vivo (fig. 4). Refeeding chicks following a fast produced quite different results in vivo (present study) from those reported in vitro (12). Fatty acid synthesis, as measured in vivo or in vitro, increased to well above control values for nonfasted chicks after refeeding for 1 day, and, as shown in figure 3, increased still further in vivo after day 2 of refeeding. After 3 days of refeeding, however, in vivo lipogenesis had returned to control levels (fig. 3), whereas Goodridge (12) found that in vitro fatty acid synthesis was still markedly enhanced. The in vitro results demonstrate that the hepatic capacity for hyperlipogenesis was still present after 3 or 5 days of refeeding, but the in vivo observations suggest that this capacity was suppressed. Cholesterol synthesis increased severalfold upon refeeding for 1 day when measured in vitro and reached normal rates of synthesis after 3 days of refeeding (12). However, cholesterol synthesis measured in vivo remained suppressed until day 3 of refeeding (fig. 4). This would suggest that although the capacity for cholesterol synthesis began to return to normal immediately upon refeeding this capacity was suppressed in the intact animal until fatty acid synthesis had subsided. The mechanism(s) involved in the suppression of fatty acid and cholesterol synthetic capacity is not clear and requires further study. Likewise, the mechanisms by which fasting and refeeding influence fatty acid and cholesterol synthesis are not completely clear (1–4).

The changes in malic enzyme observed in the present study (fig. 5) are in excellent agreement with those reported by Goodridge (12). The parallelism between the activity of this enzyme and fatty acid synthesis suggests that malic enzyme is somehow involved in lipogenesis. It has been proposed that in the rat malic enzyme functions in the generation of NADPH for the support of reductive fatty acid synthesis (21–24). Such a role for malic enzyme in chicken liver is particularly appealing since the pentose pathway cannot supply significant amounts of NADPH in this species (25, 26). Also, isocitrate dehydrogenase, another potential source of cytoplasmic reducing power, does not seem to be involved in fatty acid synthesis in the chicken (26).

The increase in liver weight relative to body weight observed upon refeeding, as well as the decrease noted in fasting (fig. 2), is in accord with previous reports for chicks (10, 11) and rats (9). The alterations in relative liver weight are undoubtedly due primarily to changes in glycogen and water content, as noted for the rat (27, 28). Summers and Fisher (11) and Feigenbaum and Fisher (10) reported that fasting did not increase liver fat in the chick, but refeeding following a fast did lead to an increased hepatic lipid content. The increase in liver fat induced by refeeding in this study was much less and was more transitory than that reported by Feigenbaum and Fisher (10). The reasons for this difference are not clear, but the larger chicks and the shorter period of starvation prior to refeeding used in the present studies undoubtedly contributed to the observed differences.

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Impairment of Gastrointestinal Processing of Fat and Protein by Ethanol in Rats [1]

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ABSTRACT Ethanol, administered simultaneously with corn oil and trace amounts of ^{131}I triolein to fasting rats, temporarily delayed the removal of the radioactivity from the gastrointestinal tract. This interference with fat absorption was dose-dependent; no significant delaying effect was seen with 2 g ethanol/kg body weight, whereas a retardation of absorption was observed with doses of 3 g or more of ethanol per kilogram body weight. The removal of ^{131}I triolein or ^{131}I oleic acid from the gastrointestinal tract was inhibited to about the same degree, indicating that blocking of intestinal lipase activity by ethanol was not an important factor in this interference. Administration of alcohol also led to retention of a protein meal in the stomach.

Ingestion of ethanol prior to, or during, a high fat meal has been reported to enhance the usual postprandial lipemia in man (1–3) and animals (4, 5). Administration of a corn oil-ethanol mixture to rats was followed by a marked increase in plasma triglyceride levels reaching peak values about 16 hours after the meal (4, 5). The plasma triglyceride rise was less pronounced with corn oil alone and the maximal level was reached after 4 to 6 hours (5).

Data from subsequent experiments have suggested that the observed delay in reaching peak triglyceride levels might have been due to an ethanol-induced impairment of gastrointestinal activity (5). The results of the present study provide more quantitative information regarding the inhibitory effects of ethanol on gastrointestinal processing of nutrients in rats.

METHODS AND PROCEDURES

Female rats of the Sprague-Dawley strain, weighing 200 to 250 g each, were studied in several different series.

Series 1. Ten animals fasted overnight were given, by stomach tube, 5 g corn oil containing trace amounts of ^{131}I triolein [2] and 5 g ethanol/kg body weight. (Corn oil containing ^{131}I triolein will be designated henceforth *I corn oil.) Examination of the ^{131}I triolein by thin-layer chromatography (6) indicated that more than 95% of the radioactivity was associated with the triglyceride moiety. Ten control animals received similar amounts of *I corn oil and dextrose in amounts isocaloric with the alcohol. No additional food was allowed; drinking water was offered ad libitum. All animals were given 0.1 ml of saturated solution of potassium iodide subcutaneously 24 hours before administration of the isotope to prevent accumulation of ^{131}I in the thyroid. Whole-body radioactivity of each animal was measured in an Armac Scintillation Detector, model no. 440,[3] 16 hours after administration of the ^{131}I-labeled oil. The animals were then anesthetized with ethyl ether and their gastrointestinal (GI) tract (stomach and intestine) with its contents, were removed. The ^{131}I activity of the GI tract and of the remaining carcass was measured in the scintillation detector, and in this series, expressed as percentage of the administered dose.

To determine the amount of ^{131}I excreted in feces and urine, eight additional rats were kept in metabolic cages. Four were given *I corn oil-alcohol mixture, and the remaining four *I corn oil alone. The ^{131}I activity was then measured in the collected feces and urine.

Series 2. The time course in the intestinal absorption of oil was studied in this series. Groups of three rats each received, by stomach tube, 5 g *I corn oil

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[2] Raolein-131, Abbott Laboratories, Chicago, Ill.

[3] Packard Instrument Company, LaGrange, Ill.

and 5 g ethanol/kg body weight and were killed 2, 4, 8, 16 or 24 hours later. As in the previous study, the control animals received *I corn oil plus dextrose. The ^{131}I activity of the whole body was measured just prior to killing. The distribution of the whole-body ^{131}I activity between the GI tract and the carcass was then determined. Blood samples were obtained by heart puncture 2, 4, and 8 hours after the meal for blood ethanol analysis (7).

Series 3. The effect of the ethanol dose on the delay in oil absorption was studied. Groups of five fasted rats each received, by stomach tube, 5 g *I corn oil/kg body weight and 2, 3, 4 or 6 g ethanol/kg body weight. The control group received similar amounts of *I corn oil and dextrose isocaloric with the 6 g/kg body weight alcohol dose. Whole-body ^{131}I radioactivity 16 hours after the treatment, and the radioactivity in the GI tract and carcass, were measured as above.

Series 4. This series was designed to determine whether the ethanol-induced delay in absorption of dietary fat might be due to an inhibition of triglyceride lipolysis, which usually precedes the process of fat absorption. Two groups of five rats each received 5 g corn oil and 5 g ethanol/kg body weight. The corn oil contained either triolein ^{131}I or oleic acid ^{131}I.[4] Two control groups received corn oil with the labeled materials and dextrose isocaloric with ethanol. The whole-body ^{131}I content 16 hours after administration of the dietary mixtures, as well as the distribution of the labeled material between the GI tract and the carcass, was measured as above.

Series 5. In the last series, five animals received, by stomach tube, 5 g egg albumin and 5 g ethanol/kg body weight. The five control animals received egg albumin only. The egg albumin contained trace amounts of ^{51}Cr-labeled bovine serum albumin.[5] Four hours later the animals were killed and the distribution of the ^{51}Cr label in the stomach, intestines, cecum and remaining carcass was measured in the Armac scintillation detector.[6]

The amounts of alcohol, oil and protein used in the present study were well tolerated by the animals. Following the dose of 5 or 6 g ethanol/kg body weight, the animals were somnolent for several hours, but responded to tactile stimuli. The relatively mild reaction to these doses of ethanol probably can be explained 1) by a higher activity of alcohol dehydrogenase in the rat, amounting to 300 mg alcohol/hour and kilogram body weight (8, 9) as compared with 100 mg/hour per kilogram body weight for man (9), and 2) by the modifying effects of the simultaneously administered fat or protein (10). None of the animals developed diarrhea.

RESULTS

The results of series 1 are shown in table 1. The reduction of the ^{131}I activity in the GI tract was taken as a measure of fat absorption, since the labeled triolein appears to be metabolized to the same extent as other triglycerides and is not deiodinated to any extent within the lumen of the GI tract. (11).

Ingestion of ethanol led to a marked retention of the radioactivity in the GI tract. Sixteen hours after administration of the oil, 78.8 ± 6.6% and 13.2 ± 5.5% of the administered doses were recovered in the stomach and intestines, respectively; without ethanol, only 10.0 ± 2.0% and 12.0 ± 1.3%, respectively, remained in these organs. The radioactivity recovered in the carcass was inversely proportional to that found in the GI tract: 46.7 ± 1.4% of the whole-body ^{131}I was contained in the carcass when corn oil was ingested with glucose, whereas only 8.4 ± 1.4% was recov-

TABLE 1

Effect of ethanol on the distribution of dietary ^{131}I triolein [1]

^{131}I recovered	Corn oil + ethanol (10)	Corn oil + dextrose (10)
Carcass	8.4 ± 1.4 [2]	46.7 ± 1.4
Stomach	78.8 ± 6.6	10.0 ± 2.0
Intestines	13.2 ± 5.5	12.0 ± 1.3
Excreted [3]	1.5 ± 1.0	20.1 ± 7.6

[1] The animals received a mixture of 5 g corn oil and 5 g ethanol/kg body weight; in the control group, ethanol was replaced isocalorically by dextrose. Number of animals in parentheses.

[2] All values are means ± SE and represent the percentage of the administered dose.

[3] Iodine-131 activity excreted by four additional animals in urine and feces.

[4] Raoleic acid, Abbott Laboratories, Chicago, Ill.

[5] Chromalbumin, E. R. Squibb and Sons, New Brunswick, N. J.

[6] See footnote 3.

ered when corn oil was administered with ethanol. The relatively high ^{131}I activity of the carcass of the control *I corn oil group indicates that the ^{131}I triolein was, to a large extent, absorbed from the GI tract.

The data on the duration of the effect of ethanol on fat absorption and on the effect of ethanol doses are presented in figures 1 and 2. The animals receiving corn oil with glucose lost the ^{131}I activity from the intestinal tract at an almost uniform rate over the first 16 hours and at a slower rate thereafter. When corn oil was administered with ethanol, there was only a slight decrease in ^{131}I activity during the first 16 hours; this was followed by absorption of a large part of the radioactivity from the GI tract over the next 8 hours and its appearance in the carcass. Blood ethanol levels 2, 4, and 8 hours after the meal amounted to 210 ± 27, 214 ± 31 and 120 ± 32 mg/100 ml, respectively.

The absorption of ^{131}I labeled oleic acid and ^{131}I triolein was equally retarded by ethanol (table 2): 89 ± 2.2% of the ^{131}I whole-body counts remained in the GI tract when the ^{131}I label was supplied as triolein, and 94.2 ± 3.1% were found when the ^{131}I carrier was oleic acid. In the groups receiving corn oil without ethanol, less ^{131}I label was retained in the GI tract with oleic acid than with triolein, suggesting an increased absorption of oleic acid.

The data from the protein study (fig. 3) indicate that impairment of gastrointestinal processing by alcohol was not limited to dietary fat. The animals receiving alcohol retained most of the ^{51}Cr label in the stomach 4 hours after the protein meal. In the control animals, the ^{51}Cr label was recovered mainly in the intestinal tract.

DISCUSSION

Results obtained in the present study indicate that larger doses of ethanol administered simultaneously with corn oil delayed the absorption of the oil for at least 16 hours. This effect seemed to be dose dependent. An impairment of the gastrointestinal processes of fat began to appear with a dose of 3 g ethanol/kg body weight, and was more pronounced with the higher ethanol doses. The dose of 2 g ethanol/kg body weight appeared to be well tolerated and did not show any delaying effect on fat absorption 16 hours after the meal. A delay of shorter duration, however, cannot be excluded.

The primary effect of ethanol appeared to be a temporary inhibition of gastric emptying. Several mechanisms could have been involved in such interference with gastric activity: 1) The reported repression of gastric motility by ethanol (12, 13) was probably an important contributory factor; 2) An irritation of gastric mucosa

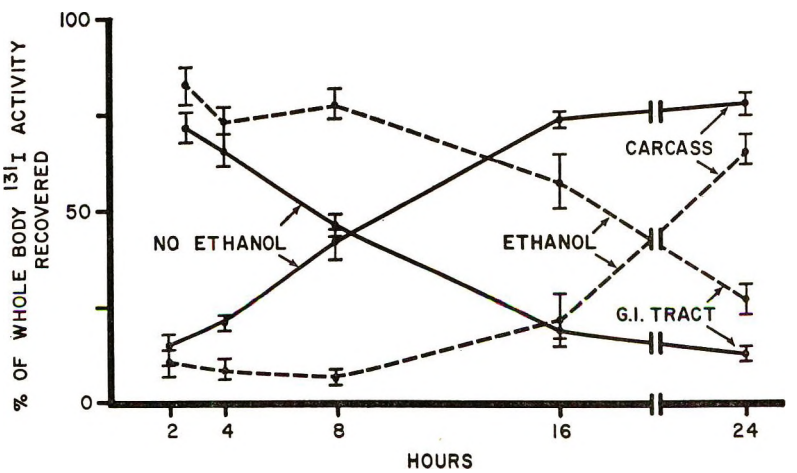

Fig. 1 Longitudinal changes in the distribution of ^{131}I label between the GI tract and carcass of rats receiving 5 g corn oil and 5 g ethanol/kg body weight. Vertical bars represent the standard error of the mean.

TABLE 2

Effect of ethanol on distribution of ^{131}I label from triolein or oleic acid

^{131}I tracer and activity in	Corn oil + ethanol [1]	Corn oil + dextrose [1]
	%	%
^{131}I triolein		
GI tract	89.0 ± 2.2 [2]	39.8 ± 4.1
Carcass	9.0 ± 1.5	43.4 ± 2.4
^{131}I oleic acid		
GI tract	94.2 ± 3.1	23.0 ± 1.5
Carcass	13.2 ± 1.8	52.0 ± 1.0

[1] The rats received 5 g corn oil and 5 g ethanol/kg body weight, or 5 g corn oil + dextrose isocaloric with ethanol.

[2] Mean ± SE, five animals per group; percentage of whole-body counts.

by ethanol and subsequent spasm of the pylorus have been also reported (14). This reaction, however, is less pronounced when food is present in the stomach (15). Furthermore, a delay in gastric emptying can be also produced in the absence of intragastric irritation, i.e., by ethanol administered intravenously or intraperitoneally (16, 17), indicating participation of additional humoral and neural factors in this process.

The severity of the ethanol-induced interference with gastric emptying was unexpected. In the protein experiment, about 90% of the ^{51}Cr dose was retained in the stomach 4 hours after the meal with ethanol; only about 5% of the tracer was found when the meal was given without ethanol.

Ethanol also has been reported to interfere with the intestinal absorption of vitamin A (18), xylose (17) and amino acids (19). The results of the present study do not allow any definite conclusions regarding the effect of alcohol on intestinal absorption of fat, although the data from a previous study (5) suggest a possible delay. Reports in the literature have indicated that ethanol might interfere with the circulating lipolytic activity (20, 21). Our observations that absorption of oleic acid was depressed to the same degree as absorption of triolein suggest, however, that either ethanol did not inhibit the intestinal lipolytic activity, or that such an inhibition did not play an important role in the intestinal fat absorption.

ACKNOWLEDGMENTS

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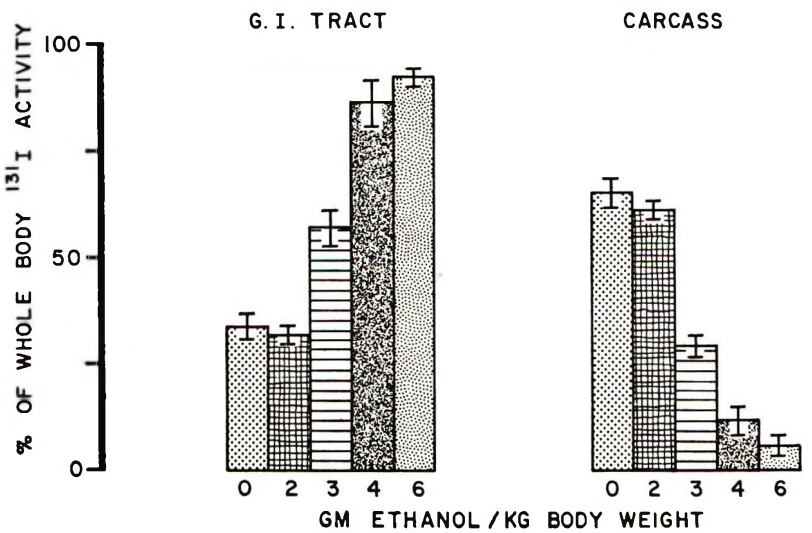

Fig. 2 Effect of ethanol dose on ^{131}I activity in the GI tract and carcass of rats receiving ^{131}I triolein and fat 16 hours earlier. Vertical bars represent the standard error of the mean.

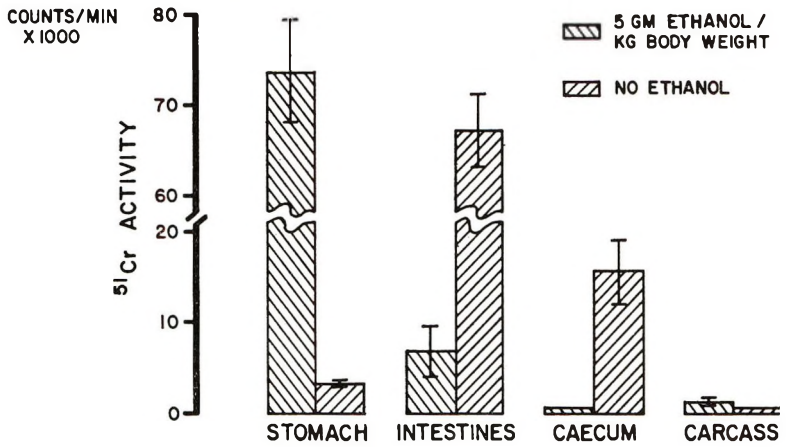

Fig. 3 Effect of ethanol on absorption of protein labeled with ^{51}Cr.

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Invitation for Nominations for 1970 American Institute of Nutrition Awards

Nominations are requested for the 1970 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1969,* to be considered for the 1970 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

Borden Award in Nutrition [1]

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Foundation Inc. The Award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritional significance of any food or food component. The Award will be made primarily for the publication of specific papers during the two previous years but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time. Employees of Borden Inc are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman
1967 – R. H. Barnes
1968 – C. L. Comar
1969 – H. P. Broquist

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R. T. HOLMAN, *Chairman*
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A. L. BLACK

Send nominations to:
R. T. HOLMAN
Hormel Institute
University of Minnesota
Austin, Minnesota 55912

Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation,

[1] Sponsors of nominees for this award should note that a change has been made in the area of research which this award recognizes.

Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made a most significant recent contribution or has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Individuals who have received another award provided by the Nutrition Foundation are not eligible for this award, unless the new award is clearly for research and contributions different from that of the first award.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
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1965 – D. M. Hegsted
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1968 – C. H. Hill
1969 – H. N. Munro

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H. N. MUNRO

Send nominations to:

M. K. HORWITT
St. Louis Univ. School of Medicine
St. Louis, Missouri 63104

Mead Johnson Award for Research in Nutrition [2]

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 40th birthday at the time the award is presented. Selection by the Jury of Award will be based either on a single outstanding piece of recent research in nutrition or on a series of recent papers on the same subject.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane
1967 – W. N. Pearson
1968 – H. F. DeLuca
1969 – R. H. Wasserman

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Send nomination to:

H. E. SAUBERLICH
U. S. Army Medical Research and Nutrition Laboratory
Fitzsimons General Hospital
Denver, Colorado 80240

Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

Former recipients of this award are:

1966 – C. Glen King
1967 – J. B. Youmans
1968 – W. H. Sebrell, Jr.
1969 – F. J. Stare

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Send nominations to:

OLAF MICKELSEN
Department of Foods & Nutrition
Michigan State University
East Lansing, Michigan 48823

[2] Sponsors of nominees for this award should note the change which has been made in the age limitation.

Invitation for Nominations for 1970 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

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Cornell University
Ithaca, New York 14850

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J. B. Brown (1964)
Thorne M. Carpenter (1958)
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Casimir Funk (1958)
Wendell H. Griffith (1963)
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C. Glen King (1963)
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Karl E. Mason (1969)
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Agnes Fay Morgan (1959)
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Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

L. C. NORRIS, *Chairman*
A. E. SCHAEFER
P. L. DAY

Send nominations to:

L. C. NORRIS
Department of Poultry Husbandry
University of California
Davis, California 95616

The following persons have been elected previously as Honorary Members of the Society:

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W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Dame Harriette Chick
F. W. A. Clements
Hans D. Cremer
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Karl Guggenheim
Egon H. Kodicek
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