Preliminary Study of Carbohydrates in the Urine of Manganese-deficient Guinea Pigs at Birth [1]

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ABSTRACT Carbohydrate components of urine of manganese-deficient and control newborn guinea pigs have been determined before the animals had access to extrauterine nourishment. Small amounts of the sugars arabinose, fructose, fucose, galactose, glucose, lactose, mannose, ribose and xylose were found in the urine of newborn guinea pigs of both manganese-deficient and control dams. Equating urine specimens on the basis of creatinine concentration revealed that slightly higher amounts of ribose occurred in the manganese-deficient progeny. The most striking difference in the carbohydrate components of urine was a threefold higher concentration of free myoinositol in the urine of control animals at birth. Compounds were determined by paper chromatography techniques. Some speculation is made about the reduced myoinositol content of urine of manganese-deficient young, the synthesis of glucuronic acid in fetal development, and the relation of these to connective tissue defects known to occur in manganese-deficient guinea pigs.

Earlier studies of the influence of a maternal deficiency of manganese in guinea pigs have revealed that the progeny have skeletal abnormalities at birth which are believed due to defective cartilage matrix (1). The acid mucopolysaccharides present in rib and epiphyseal cartilage were significantly reduced in the manganese-deficient animals. More recent studies have shown that the young of guinea pigs receiving low intakes of manganese also have abnormalities of the pancreas. Animals which survived only a few hours had aplasia or marked hypoplasia of pancreatic tissue (2). Some congenitally deficient animals continued on the low manganese diet survived to adult age, and when these animals were given glucose intravenously, glucose tolerance was found to be abnormal (3).

In the present study free sugars and sugar alcohols have been investigated in bladder urine of manganese-deficient and control guinea pigs at birth, as one means of exploring possible metabolic variations in the manganese-deficient animals.

The urinary excretion of sugars has been determined by several groups of investigators, and information available at this time deals mainly with the excretion of sugars by human subjects. Tower and his associates (4, 5) have investigated the excretion of pentoses in neuromuscular diseases; Woolf and Norman (6) have reported lactose, galactose, glucose, fructose, arabinose and xylose present in urine in early infancy; Howarth and Macdonald (7) have investigated reducing sugars in the urine of premature babies; and Bickel (8) has studied mellituria in both infants and children. The excretion of several sugars occurred more often in infants under 10 days of age, and interpretations of their presence in urine included lack of kidney function in the young infant and immature liver function. The origin of some of these sugars was uncertain.

Great progress has been made in the past 15 years on knowledge of the occurrence of carbohydrates in tissues and their participation in carbohydrate-protein linkages. Glegg et al. (9) reported the presence of galactose, glucose, mannose and fucose in reticular fibers, suggesting that these fibers contain a carbohydrate-protein complex. Neutral sugars present in fetuin of fetal calf serum include galactose and mannose in the ratio of 3:2 (10). Recent work has revealed that galactose and xylose are linked with serine forming a carbohydrate-protein linkage of heparin

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and protein (11). Chondroitin 4-sulfate from bovine nasal septa has been investigated and was found to be linked to protein through a glycosidic linkage between xylose and serine, with galactose also present as a constituent of the carbohydrate-protein complex (12). Arabinose- and xylose-containing complexes in human brain were first reported by Stary et al. in 1962.[2] Two distinct polysaccharide peptides, a hyaluronate peptide containing arabinose and a chondroitin sulfate peptide containing xylose, were identified from protease-digested brain preparation in 1966 (13).

Carbohydrates appear to play an important role in the development of many tissues, and it was, therefore, believed worthwhile to determine their occurrence in the urine and tissues of newborn guinea pigs born to control and manganese-deficient dams. Preliminary data on the sugar and sugar alcohol components of urine are reported at this time.

MATERIALS AND METHODS

Information dealing with rations and general procedures for studying manganese deficiency in the guinea pig have been described in former papers (1, 14). Low manganese rations provided less than 3 ppm of the trace element, and control animals received 125 ppm manganese as the sulfate.

At autopsy immediately after birth, it was frequently observed that the bladders of guinea pigs were greatly distended and that 0.5 to 5.0 ml of urine could be collected by syringe. Urine specimens were collected in this manner before the young received extrauterine nourishment. Six pooled samples of urine were prepared for the present study representing urine from a total of 9 manganese-deficient animals and 12 controls. All deficient animals tested had severe signs of manganese deficiency based on their ataxic condition. The average birth weight of deficient animals was 111 g and control young averaged 113 g. Each of the pooled urine preparations was diluted to 25 ml, and duplicate aliquots were removed for creatinine determinations (15). Pooled urine preparations were treated with urease for 24 hours at 5°. Each was then concentrated in a rotary evaporator at 35° and reduced to a volume of 3–5 ml. The total concentrated preparation was desalted by passing one-half portions, adjusted to pH 5, over resin columns containing Dowex 50-X8 (200–400 mesh), hydrogen form. Carbohydrates were removed in 100 ml of boiled deionized water using a flow rate of 1 ml/3 minutes. The combined eluate for each pooled urine preparation was then concentrated to a volume of 2 ml by means of a rotary evaporator and was next deproteinized using zinc sulfate and barium hydroxide. The protein-free filtrate was concentrated to dryness in a vacuum oven, and the carbohydrates were taken up in pyridine by heating at 100° for 10 minutes. After the pyridine was removed by drying in a vacuum oven, the final product was dissolved in 2 ml of 10% isopropyl alcohol. Preliminary tests, carried out on comparable urine preparations, revealed that aliquots equal to 160 μg of creatinine were satisfactory for most chromatograms.

Descending paper chromatography,[3] using the following solvent systems was employed to separate urine components: Ethyl acetate–pyridine–water (40:11:6); phenol saturated with water (80:20); *n*-butanol–pyridine–water (6:4:3); and isopropyl alcohol–ammonia–water (70:10:20). For separation of mannose, fructose and arabinose, flavognost [4] was added to the ethyl acetate–pyridine–water solvent mixture at the rate of 140 mg/100 ml (16). Detecting procedures included silver nitrate, triphenyltetrazolium chloride, aniline hydrogen phthalate, *p*-anisidine·HCl and phloroglucinol solution (16). Because of the number of spots encountered in the urine specimen chromatograms and the wide difference in R_F values of the carbohydrate components, running times using the ethyl acetate–pyridine–water (40:11:6) solvent mixture varied from 22 hours to 400 hours. Standards of the various compounds present in urine were mixed with the prepared samples and also spotted separately and simultaneously for each paper chromatogram. Procedures for

[2] Stary, Z., S. Terkman and N. Oner 1962 Proc. Amer. Chem. Soc., Fourth Delaware Valley Regional Meeting, abstracts of papers, p. 42.

[3] Whatman no. 1 filter paper no. 28451-1, Van Waters and Rogers, Incorporated, San Francisco, California.

[4] Diphenylboric acid, ethanolamine complex, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

expressing carbohydrate components of urine on a semiquantitative basis consisted of comparing five concentrations of the standard with the spotted urine sample and judging concentrations on the basis of visual comparisons and densitometer recordings.[5]

RESULTS

As a result of preparing numerous paper chromatograms using the ethyl acetate–pyridine–water (40:11:6) solvent mixture and applying urine preparations equivalent to as much as 640 μg creatinine, it was found that ribose was the fastest migrating carbohydrate present. As seen in table 1 and figure 1, the urine of manganese-deficient guinea pigs contained a somewhat larger amount of ribose. This was observed for each of the three pooled urine preparations tested. A total of 9 sugars: arabinose, fructose, fucose, galactose, glucose, lactose, mannose, ribose and xylose have been tentatively identified by their mobility rates and reactions to the various detecting agents used. Quantities of sugars present in the urine of deficient and control animals at birth were much alike except in the case of ribose. Satisfactory separation of mannose and fructose was accomplished by the use of flavognost,[6] and arabinose also separated from the two sugars under these conditions. By lengthening the running time and using the same solvent mixture (ethyl acetate–pyridine–water, 40:11:6), good separation was obtained for xylose and fucose and between glucose and galactose.

Slowly migrating carbohydrate components were first studied using ethyl acetate–pyridine–water and by increasing the running time to 200 hours and up to 400 hours. Figure 2 illustrates the migration of standard myoinositol under these conditions and the comparative amounts of myoinositol in the urine of manganese-deficient and control guinea pigs at birth. Of the three sets of urine specimens tested, the manganese-deficient sample in each case contained less myoinositol than the control specimen, varying from one-half to one-fourth the amount of the cyclitol found in matching control animals.

Spots tentatively identified as ribose and myoinositol by one-dimensional chromatography were removed and rechromatographed to investigate the possible presence of more than one compound. Findings were negative. Earlier work which included tests of inositol in urine, nerve and testis had revealed that the compound migrating similarly to standard myoinositol stimulated growth of *Saccharomyces cerevisiae* when added to an inositol-free medium. To date, quantitative differences in the myoinositol content of urines of manganese-deficient and control guinea

[5] Densicord, densitometer model no. 520 with 42B recorder and integrator model 49, Photovolt Corp., New York, New York.
[6] See footnote 4.

TABLE 1

Carbohydrate components of urine of guinea pigs at birth

Standard compound	Comparative information of the amounts of carbohydrate components of urine in manganese-deficient and control guinea pigs at birth based on one-dimensional chromatography tests
D (−) Ribose	2 × quantity in manganese-deficient animals
D (+) Xylose	No distinguishable difference
L Fucose	No distinguishable difference
D (−) Arabinose	No distinguishable difference
D (−) Fructose	No distinguishable difference
D (+) Mannose	No distinguishable difference
D (+) Glucose	No distinguishable difference
D (+) Galactose	No distinguishable difference
Unidentified	No distinguishable difference
Unidentified	No distinguishable difference
Myoinositol	3 × quantity in control animals
Unidentified	Higher amount in manganese-deficient animals
Unidentified	Uncertain
Unidentified	Higher amount in manganese-deficient animals

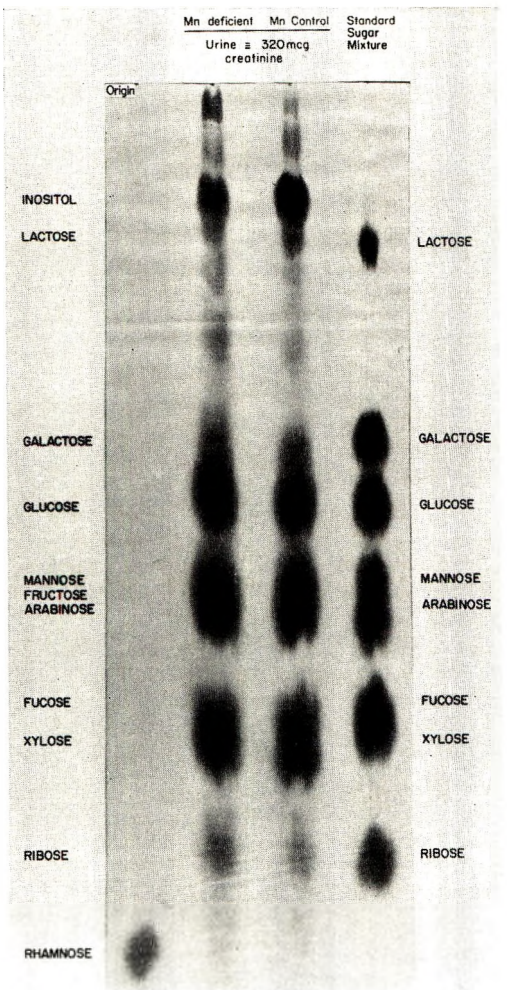

Fig. 1 Paper chromatogram with standard sugar mixture (right) and urine preparations equivalent to 320 μg creatinine. Ethyl acetate–pyridine–water, 40:11:6. Running time 22 hours. Silver nitrate detection.

pigs at birth have been shown by paper chromatography alone.

The identity of additional slowly migrating components, particularly the prominent spot shown in figure 2 for urine of manganese-deficient animals, has not been tested adequately to report at this time and must await additional test material.

DISCUSSION

The decision to explore metabolic differences in the newborn guinea pig by analyses of urinary components posed the question of the suitability of using creatinine excretion as a basis for comparing animals of the two ration groups. Much information dealing with creatinine excretion by subjects of various ages has been reviewed by Peters and Van Slyke (17). There appears to be general agreement that creatinine excretion in infants bears the same relation to muscle mass that it

Fig. 2 Paper chromatogram with standard myoinositol (left) and urine preparations equivalent to 160 μg creatinine. Ethyl acetate–pyridine–water, 40:11:6. Running time 200 hours. Silver nitrate detection.

does in adults. Therefore, creatinine elimination was accepted as the best available method of comparing carbohydrate components of the urine at birth. Manganese deficiency per se has not been found to influence creatinine excretion.

The finding that manganese-deficient guinea pigs at birth excreted approximately one-third the myoinositol found in the urine of control animals is believed to be of importance and may identify a handicap of metabolism during fetal growth.

One of the functions of myoinositol is that of a precursor of free glucuronic acid (18) which is needed for synthesis of supporting structures. The second precursor, uridine diphosphate glucuronic acid, is dependent on glucuronyl transferase to yield the free glucuronic acid and also requires UDPG dehydrogenase to form from UDP-glucose (18). Brown et al. (19) have studied the activity of both enzymes in fetal, newborn and adult guinea pig liver. They state, "Defects have been demonstrated in two enzymatic steps in the glucuronide-synthesizing system in the fetal and newborn guinea pig. The glucuronyl transferase activity as well as the UDPG dehydrogenase activity are markedly deficient in the fetus and gradually increase during the first few days of life." Dutton (20, 21) has also reported the early fetal guinea pig liver to have negligible glucuronide-synthesizing capacity.

The extent to which each of the two pathways for formation of glucuronic acid functions during the progressive development of the fetal guinea pig is not known; however, it seems reasonable that myoinositol may play a more important part in the synthesis of glucuronic acid and in the ultimate synthesis of supporting structures than is generally recognized. If the reduced concentration of myoinositol in the urine of manganese-deficient guinea pigs at birth is due to an inhibition in the synthesis of the cyclitol, it will be of great interest to know where this block occurs. The studies of Chen and Charalampous (22) and Eisenberg (23, 24) will be especially helpful in determining whether there is inhibition in cyclization of glucose 6-phosphate to *d*-myoinositol 1-phosphate or in the conversion of the latter to free myoinositol by a highly specific phosphatase. These speculations offer many challenges for the future.

The appearance of 9 sugars in the glomerular filtrate of both manganese-deficient and control guinea pigs at birth is believed to reflect both the participation of these carbohydrates in tissue synthesis of this species at birth and some defect in tubular reabsorption. The significance of the small differences in ribose noted earlier is unknown and should have additional study.

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Rat Assay of a Simulated Human Diet for Antithyrotoxic Substances [1]

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ABSTRACT Since certain dietary substances markedly alter the turnover and utilization of thyroid hormone in the rat, it is important to determine whether the same substances also affect thyroid hormone metabolism in the human. To conduct such a study, it is necessary to devise a diet that is very low in antithyrotoxic substances, and to find suitable foodstuffs that are high in antithyrotoxic activity. To this end, a simulated human diet was prepared and fed to rats given exogenous thyroxine (T_4). The diet was very low in antithyrotoxic activity, and the inclusion of liver, milk, beef and fish into the diet afforded the same protection against the physiological effectiveness of T_4 as liver residue, a commercial liver preparation, known to have high antithyrotoxic substance activity. Based on these results, it should be possible to prepare human diets both low and high in antithyrotoxic substance activity, and to determine the importance of these substances in human thyroid hormone metabolism and economy.

The mechanism by which certain dietary substances interfere with the peripheral activity of thyroid hormone in the rat is now fairly well established (1, 2). Diets containing substances such as liver, fish, milk, cottonseed meal, and soybean meal products are effective in blocking the usual physiological response to exogenous thyroid hormone in the rat (3, 4). To this list can also be added alfalfa hay, alfalfa grass silage (5), cellulose and laboratory ration (6). It is not known whether all contain the same antithyrotoxic factor, or whether they act by the same mechanism. Crude bovine hemoglobin,[2] liver and cottonseed meal, however, produce a marked increase in the fecal excretion of thyroxine (T_4) and a subsequent reduction in the circulating blood level of either injected or orally administered T_4 (1, 2). The increased fecal excretion of T_4 is probably the result of at least three contributory factors: 1) the antithyrotoxic substances (ATS) cause an oral dose of T_4 to move rapidly through the lower ileum which is an active site for T_4 absorption; 2) the fecal mass is larger in animals fed ATS and this may be responsible for the increased intestinal motility; and 3) it is believed that ATS bind T_4 into a relatively unavailable complex. The evidence for the latter concept is threefold. First, the feces from rats fed hemoglobin (Hb) contain little biologically active T_4 (1); second, known antithyrotoxic substances inhibit the uptake of ^{131}I-labeled T_4 by the mucosa of intestinal sacs made from the ileum (1); and third, Hb forms an inactive complex with T_4 and interferes with the catalytic role of T_4 in the oxidation of NADH by the horseradish peroxidase system (7). Although ATS are more effective against an oral dose of thyroxine, they also interfere with the activity of injected thyroxine. The latter observation suggests that ATS interfere with the enterohepatic recycling of thyroid hormone by binding thyroxine into an unavailable complex and causing it to be excreted into the feces. Consequently, ATS prevent the serum protein-bound iodine from reaching levels that would otherwise produce the usual physiological responses to T_4.

The rat is known to have a much larger enterohepatic circulation than the human (8) and whereas ATS are very important to thyroid hormone metabolism in the rat, they may have little importance in the human. Before human studies can be undertaken, however, it is necessary to

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devise suitable diets that are extremely low in ATS and to find palatable foodstuffs that are rich in such substances.

In the present study, two sample menus or diets for human use were devised, and from these a single diet was prepared and tested for ATS activity in the rat assay system. The diet was found to have little or no ATS activity and the addition of certain foodstuffs rich in ATS afforded good protection against the physiological responses that usually accompany the administration of T_4. Consequently, palatable human diets low in ATS activity could be prepared using the foodstuffs tested in the present study.

TABLE 1
Sample menus low in ATS activity that could be used in human studies

Menu no. 1	Menu no. 2
Breakfast	
Orange juice	Apple juice
Cream of Wheat	Oatmeal
Sugar	Sugar
Coffee	Coffee
Noon meal	
Pineapple juice	Chicken soup and crackers
Chicken (white meat)	Chicken (white meat)
Cooked rice	Carrots
Wax beans	Jello
Peaches	Corn pone
Jello	Jelly
Coffee	Coffee
Sugar	
Evening meal	
Grape juice	Tomato juice
Turkey (white meat)	Turkey (white meat)
Boiled potato	Mashed potato
Corn oil	Beets
Peas	Corn oil
Pears	Corn muffin
Corn muffin	Applesauce
Coffee	Coffee
Sugar	
Evening snack	
Apricot nectar	Grape juice
Crackers	Crackers
Jelly	Honey or jelly

METHODS

Male rats of the Sprague-Dawley strain weighing 150 ± 10 g, were maintained in a constant-temperature room (25°) with controlled lighting (6 AM to 6 PM). Fresh food and water were offered daily ad libitum and stocks of all diets were kept in the frozen state prior to their use.

Solutions of L-thyroxine (T_4) which were administered daily by gavage were prepared by dissolving T_4 in a 1% (w/v) sucrose solution adjusted to a pH of 8 to 9. At the end of the twelve-day feeding period, metabolic rate determinations were made twice on each animal by a procedure described previously (9). All animals were decapitated and liver samples were homogenized and assayed for mitochondrial α-glycerophosphate dehydrogenase (GPD) activity (1, 2).

Two menus thought to be low in ATS were devised by excluding all foodstuffs previously shown to have antithyrotoxic activity in the rat (3, 4, 10–13). From these (table 1), a single diet was prepared (table 2) and fed to the rats. All the dry ingredients listed in the upper portion of table 2 were finely ground and mixed together in a large food mixer. The meat products were obtained fresh and cooked in a pressure cooker. The cooked meats were finely ground, and together with the juices were added to the dry mix, and thoroughly blended. Finally, the fruits and vegetables (as baby food purees) were added, followed by the concentrated fruit juices, and other items shown in the bottom portion of table 2. To assure an adequate vitamin intake, both the water-soluble and fat-soluble vitamins shown in the legend of table 2 were also added to the diet. When completely mixed; the diet had a rather thick consistency, it was divided into small portions and frozen. Fresh food was supplied daily, and all food remaining from the previous day's feeding was discarded. Those foodstuffs rich in ATS (liver, beefsteak, whole milk powder and fish) were substituted for the white meat of chicken and turkey (50:50 mixture) at the levels indicated in table 2.

EXPERIMENTAL

Experiment 1. Of the five groups of eight animals each employed in this experiment (table 3), the first three received the low-ATS diet. Group 1 received the diet alone; group 2 was given daily doses of 50 μg of T_4 per 100 g body weight by gavage and group 3 received the same dose lveel of T_4 plus 6% (w/w) liver residue [3]

[3] Purchased from Wilson and Company, Chicago, Illinois.

TABLE 2

Composition of the rat diets

	Low ATS diet	High ATS diet	Protein	Water
	g/100 g	*g/100 g*	*g*	*g*
Oatmeal	4.4	4.4	0.62	0.37
Cream of Wheat	4.4	4.4	0.50	0.45
Coffee (concentrate)	1.0	1.0	—	0.03
Corn meal muffin mix	6.6	6.6	0.41	0.51
Rice	4.4	4.4	0.29	0.33
Crackers (soda)	1.0	1.0	0.09	0.04
Jello (assorted)	2.3	2.3	0.22	0.30
Potatoes (powdered)	6.0	6.0	0.51	0.43
Corn meal	4.4	4.4	0.41	0.53
Sugar	1.2	1.2	—	0.01
White meat of chicken and turkey (pressure cooked)	45.7	—	12.91	29.16
Liver (pressure cooked)	—	13.8	3.60	7.73
Beef steak (pressure cooked)	—	13.8	3.40	8.10
Whole milk powder	—	9.1	2.40	0.18
Fish (haddock, pressure cooked)	—	9.0	1.67	6.03
Canned peaches [1]	2.5	2.5	—	1.95
Beets	1.5	1.5	0.02	1.34
Peas	1.5	1.5	0.05	1.28
Wax beans	1.5	1.5	0.02	1.39
Carrots	1.5	1.5	0.01	1.37
Corn oil	0.5	0.5	—	—
Concentrated fruit juices	6.6	6.6	0.15	3.84
Jelly (assorted)	1.0	1.0	—	0.29
Applesauce	2.0	2.0	—	1.62
Vitamins [2]				

[1] All canned fruits and vegetables were obtained as purees (baby foods).

[2] A complete vitamin mix was added which supplied the following amounts of vitamins: (mg/100 g diet) choline chloride, 100; inositol, 50; thiamine·HCl, 5; riboflavin, 5; niacin, 10; pyridoxine·HCl, 1.5; folic acid, 0.5; pantothenate, 20; p-aminobenzoic acid, 10; menadione, 0.1; vitamin B_{12}, 0.01; mixed tocopherols, 60; cod liver oil, 600; and biotin, 0.05.

TABLE 3

Summary of data obtained in the first experiment

Group no.	Regimen	Avg 12-day wt gain	Liver GPD [1]	MR
		g		
1	Low ATS diet (controls)	77.5 ± 4.3 [2]	19 ± 1.4	8.3 ± 0.4
2	Low ATS diet + 50 μg T_4	61.2 ± 3.8	333 ± 2.3	13.0 ± 0.7
3	Low ATS diet + 6% LR + 50 μg T_4 [1]	67.5 ± 4.4	91 ± 5.4	10.4 ± 0.3
4	High ATS diet (controls)	81.9 ± 3.7	21 ± 2.4	7.9 ± 0.3
5	High ATS diet + 50 μg T_4	75.8 ± 4.0	120 ± 4.4	10.2 ± 0.2

[1] Liver GPD activity has the units of μliters O_2/10 minutes/150 mg fresh liver and MR (metabolic rate) has the units of liters of O_2/m^2/hour. T_4 was administered daily by gavage at a dose level of 50 μg/100 g body weight, and LR (liver residue) was included in the diets at a level of 6 g/100 g diet.

[2] Mean ± SE.

added to the diet. Group 4 was fed the high ATS diet without T_4, the group 5 received the same diet plus daily doses of 50 μg T_4 per 100 g body weight by gavage.

Both the low ATS diet (group 1, table 3) and the high ATS diet (group 4) produced the same amount of growth ($P = 0.45$). The administration of 50 μg T_4 produced no growth depression in animals receiving the high ATS diet (group 5), but animals receiving T_4 on the low ATS diet (group 2) showed a significant reduction in growth ($P = 0.02$).

TABLE 4
Summary of data obtained in experiment 2

Group no.	Regimen	Avg 12-days wt gain	Liver GPD [1]	MR
		g		
1	Low ATS diet (controls)	72.6 ± 3.1 [2]	27 ± 2.9	8.1 ± 0.3
2	Low ATS diet + 25 μg T_4	72.5 ± 4.8	170 ± 4.1	10.0 ± 0.3
3	Low ATS diet + 6% LR + 25 μg T_4 [1]	87.0 ± 5.9	66 ± 3.7	8.2 ± 0.3
4	High ATS diet (controls)	67.3 ± 3.5	27 ± 2.1	7.3 ± 0.2
5	High ATS diet + 25 μg T_4	71.2 ± 4.1	52 ± 4.2	8.4 ± 0.3

[1] The abbreviations and units used are the same as those given in table 3.
[2] Mean ± SE.

The dose level of T_4 used in this experiment produced a marked liver GPD response and metabolic rate (MR) response which was considerably reduced either by liver residue feeding or by the substitution of beef, liver, milk and fish for the chicken and turkey component of the diet. Neither liver residue nor the natural foodstuffs reduced the GPD and MR values to control levels, and liver residue was slightly more effective in lowering the GPD response than the natural foodstuffs ($P = 0.001$).

Experiment 2. Because the level of T_4 used in the previous experiment was rather high, the experiment was repeated, using a lower dose level of T_4. Five groups of eight animals each were fed the same diets as before (table 4). Group 2, 3, and 5 received twenty-five μg of T_4 per 10 g body weight/day by gavage for twelve days, at the end of which time the same measurements were made as in experiment 1.

This dose level of T_4 produced a much smaller GPD and metabolic rate response, and both liver residue and the natural foodstuffs containing ATS activity were about equally effective in counteracting these responses (table 4). Neither source of ATS activity, however, reduced the liver GPD to control levels.

DISCUSSION

Irrespective of the dose level of T_4 employed, the basic low ATS diet appears to be quite low in ATS activity, as evidenced by the large liver GPD and MR responses obtained. These responses are comparable to those seen in animals receiving a purified sucrose–casein–corn oil type of diet (13, 14). The substitution of beef, liver, milk and fish for the white meat of chicken and turkey affords approximately the same protection against the physiological activity of T_4 as liver residue, a substance known to have considerable ATS activity (2, 9). Both sources of ATS activity kept the metabolic rate at the same level (tables 3 and 4). The high ATS diet, however was not quite as effective as 6% liver residue (table 3) in suppressing the GPD response to 50 μg T_4 ($P = < 0.02$), but it was slightly more effective than liver residue when 25 μg T_4 was given ($P = < 0.02$). Changes in body weight are a poor and inaccurate measure of a rat's response to thyroid hormone, and liver GPD gives a more specific and sensitive response than the metabolic rate since the latter is influenced by many factors other than thyroid hormone (15).

In view of the fact that the basic low ATS diet is low in ATS activity, and that beef, liver, milk and fish appear to contain appreciable amounts of ATS activity, it should be possible to prepare suitable diets for human consumption that would be either very low in ATS activity or relatively rich in such activity. Each human subject might serve as his own control in determining the importance of the antithyrotoxic substances in thyroid function. The subject could receive a low ATS diet prior to a study of his turnover and excretion of a tracer dose of labeled T_4. Then, the labeled T_4 study could be repeated after the subject had received a diet rich in ATS for a comparable period of time. If human subjects show an increased excretion of labeled T_4 on the rich ATS diet, then dietary ATS may well be important in human thyroid function and metabolism.

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Effect of Graded Levels of Cadmium on Tissue Uptake of ^{65}Zn by the Chick Over Time [1]

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ABSTRACT To ascertain if part of the interference of dietary cadmium with the utilization of dietary zinc might be due in part to competition for sites on a blood transport system, and to detect other early interference, chicks were fed graded levels of cadmium with each of three levels of a single dose of ^{65}Zn. Uptake by the blood, liver and tibiae was measured over a 24-hour period. Interference with uptake of 3 μg of ^{65}Zn was not found when zero, 5, 10 or 50 ions of Cd (mole equivalents) were included in the dosage. When a basal dose of 18 or 27 μg of ^{65}Zn was fed, 5 or 10 molEq of cadmium caused a marked decrease in absorption of ^{65}Zn. The difference in blood levels persisted over 24 hours, suggesting that cadmium was occupying some of the blood-binding sites. The ^{65}Zn accumulated in the liver of the chicks fed cadmium so that uptake did not differ from the basal group from 8 to 24 hours. The increase in liver uptake was not reflected in uptake by the tibiae, the latter being significantly lower than the basal group. It is postulated that cadmium interferes with the absorption of zinc through occupation of some of the binding sites of a transport system in the blood; further interference with utilization of the absorbed zinc occurs at a more fundamental level. It is suggested that the effect of dietary cadmium on dietary zinc will depend on the absolute amounts of each, the proportions of one to the other, and the length of the feeding period.

A syndrome, characteristic of zinc deficiency, has been produced in the bovine (1) and the poult (2) by addition of cadmium to the diet. Interactions of orally fed cadmium with zinc, copper, and iron have been observed in the chick (3) and rat and mouse (4).

Much of the work on the effect of cadmium on zinc uptake by tissues has been concerned with competition between these ions when either or both were injected. Injected zinc is "loosely" bound to plasma proteins (5) which are thought to serve as transport sites for zinc in the blood (6). Homeostatic controls regulating zinc distribution in the tissues, however, are operative only when both absorptive and excretory mechanisms are acting together (7).

Zinc and cadmium behave very similarly chemically and both form tetrahedral complexes. If orally administered zinc were transported by plasma proteins, a competition between the two ions for the same binding sites on the transport system might occur when a sufficient number of both ions were present to saturate the sites. The results of the competition could be reduced absorption of zinc, the magnitude of the effect being dependent on the proportion of cadmium to zinc ions. Dietary zinc would then be less available to the chick in the presence of dietary cadmium, the symptoms of a zinc deficiency could appear even when the diet furnished supposedly adequate zinc.

Beside decreasing the absorption of zinc, interference with the use of zinc by other tissues might occur. The level of zinc in the liver of calves given cadmium was increased over that of control animals, but the calves showed signs of a zinc deficiency (1). Hill et al. (3) suggested that cadmium and zinc interact to cause disturbances at a fundamental level.

The uptake of ^{65}Zn is a dynamic process. Over time it may be considered to follow a general pattern of absorption and transport by the blood, uptake by the liver followed by excretion or other tissue dep-

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osition. Previous work (8) had shown that the uptake of ^{65}Zn over a 24-hour period, when followed at intervals of 4, 8 and 24 hours, by blood, liver or tibiae, is affected by the amount of ^{65}Zn available for absorption. The objective was to follow the uptake of ^{65}Zn in the presence of graded amounts of cadmium over time. Both the amount of uptake and the possible variations from 1) the pattern of uptake when ^{65}Zn was fully available, and 2) the pattern previously seen when the amount of ^{65}Zn available for absorption was limited, were to be considered. By following the sequential amount and pattern of uptake of the three tissues, evidence for a zinc–cadmium transport system in the blood and clues as to some of the primary sources of interference might be found.

MATERIALS AND METHODS

Factorial experiments were conducted in which each of three levels of a basic dosage of ^{65}Zn, 3 μg, 18 μg or 27 μg, was fed as a single dose. For each dosage, zero, 5, 10 or 50 times as many cadmium ions as zinc ions were included, zero, 5, 10 or 50 molEq (mole equivalents) of Cd.

The basic dosage is given as μg of ^{65}Zn. In some of the replicates of the 18- or 27-μg trials, to make the counts per minute of the basic dosage of the triplicate trials comparable, owing to the different specific activity of the several lots of ^{65}Zn used, it was necessary to dilute the ^{65}Zn with a water solution of stable $ZnCl_2$. The trials are labeled as being comprised of ^{65}Zn for convenience; those in which dilution was necessary are so designated in the tables or figures accompanying the results.

A trial was also conducted in which the uptake of 27 μg of ^{65}Zn in the presence of zero, 5, 10 or 50 molEq of calcium, as a water solution of $CaCl_2$ was included.

The uptake of ^{115m}Cd was measured in a trial in which a combination of stable cadmium and ^{115m}Cd supplied cadmium in amounts comparable to the 5, 10 or 50 molEq used with the 18 μg of the ^{65}Zn experiment. One group received the equivalent of 5 molEq of Cd as ^{115m}Cd, other groups received 5, 10 or 50 molEq of ^{115m}Cd or ^{115m}Cd + Cd in which 18 μg of stable zinc was included. The groups are designated as receiving ^{115m}Cd to differentiate them from the groups receiving stable Cd with ^{65}Zn. The proportions of stable cadmium and ^{115m}Cd are given in the footnotes to figures 4 and 5.

One-day-old meat-type cockerels were fed a corn-soybean meal starter ration for a preliminary period of 14 to 16 days. Food was then withheld for 16 hours. The chicks were force-fed gelatin capsules containing the isotope plus the various levels of competing cation, deposited on sucrose. After 4, 8 and 24 hours, the liver, left tibia and 1 ml of blood (by heart puncture) were taken. The activity of an aliquot of the liver (about 2 g), of 1 ml of blood and of the left tibia was measured by counting for 10 minutes in a well scintillation counter.[3] Total liver activity, the activity of the two tibiae, and the total blood activity, based on a blood content equivalent to 10% of the initial weight of the chick, were calculated. The data for the activity of the tissues, expressed in percent retention of the isotope given, were analyzed for variance as a factorial experiment. The effects were subjected to the F test (9). The values denoting the least significant difference (LSD) between means at $P < 0.01$ and $P < 0.05$ were calculated. In some experiments the uptake of the three tissues was added together for any one of the three time periods to give a combined tissue uptake for the specified time. This was not subjected to statistical analysis and was used only in interpretation of the individual tissue uptakes.

One trial each was made with 3 μg of ^{65}Zn + Cd, 27 μg of ^{65}Zn + Ca, or ^{115m}Cd + 18 μg of stable zinc. Three trials each were made with 18 μg or 27 μg of ^{65}Zn each, plus cadmium. Each of the three trials was analyzed separately. There was a slight interaction of cadmium by trial and the percent of uptake varied somewhat from trial to trial. However, all trends and the conclusion drawn from each trial for 18 μg or 27 μg of ^{65}Zn were the same; the averages of the three trials for each level of zinc were combined and a LSD was calculated for the combined trials.

[3] Baird-Atomic Model 709, Baird-Atomic, Cambridge, Massachusetts.

TABLE 1

Uptake of ^{65}Zn by tissues when a basal dose of 27 μg was fed with zero, 5, 10 or 50 molEq of Cd (average of three trials [1]; percentage of dose given)

Hours	4			8			24		
Cadmium [2] added	Blood	Liver	Tibiae	Blood	Liver	Tibiae	Blood	Liver	Tibiae
molEq									
0	2.51	6.25	1.75	1.69	5.07	2.06	1.39	4.03	2.31
5	1.46 **	4.81 **	1.11 **	0.97 **	4.14	1.13 **	0.89 **	3.06	1.42 **
10	1.37 **	3.74 **	0.85 **	0.94 **	4.64	1.09 **	0.86 **	3.10	1.22 **
50	0.41 **	1.44 **	0.31 **	0.35 **	2.15 **	0.34 **	0.54 **	3.53	0.63 **

[1] Trial 1: 27 μg of ^{65}Zn (0.915 μCi/mg Zn), 9 chicks/Cd level. Trials 2 and 3: ^{65}Zn (4.15 μCi/mg Zn), diluted with a water solution of $ZnCl_2$ to supply 27 μg Zn/chick and approximate the capsule counts per minute of trial 1. Fifteen chicks per cadmium level for each trial.
[2] As a water solution of $CdCl_2 \cdot 2H_2O$.
** $P < 0.01$. LSD = 0.35 for blood values; 1.34 for liver values; 0.33 for tibia values.

RESULTS AND DISCUSSION

In a preliminary study, when 27 μg of ^{65}Zn were fed with zero, 5, 10 or 50 molEq of cadmium, highly significant ($P < 0.01$) decreases in the uptake of ^{65}Zn by the blood or tibiae occurred (table 1). The chicks had been fasted for 16 hours before the single dose and the absorption and transport of the relatively large number of ions furnished by zinc and its cadmium equivalents could be slowed by the inability of the chick to metabolize the large quantities of ions available at one time. Calcium is commonly added to diets in the salt mix; to determine the effect of large numbers of another ion of physiological significance on the uptake of ^{65}Zn, zero, 5, 10 and 50 molEq of Ca were added to the basal dosage of 27 μg of ^{65}Zn. Significant differences were not found in uptake of ^{65}Zn in the presence of calcium (table 2). Apparently the general number of ions available for absorption and transport at one time did not influence uptake of ^{65}Zn.

If a competitive zinc-cadmium interrelationship were involved in the transport of these ions by the blood with a finite number of sites available, accommodations for both ions should be available at a low dosage of cadmium and zinc. If a more fundamental biochemical mechanism were involved, 50 times as many cadmium as zinc ions might exert an effect. A dosage of 3 μg of ^{65}Zn with the corresponding zero, 5, 10 and 50 molEq of cadmium was given. Significant differences in uptake by any of the three tissues were not found over the 24-hour period (table 3). Apparently enough transport sites in the blood were available to bind both zinc and cadmium, and the presence of 50 times as many cadmium ions did not affect mechanisms governing uptake of the relatively low dosage of ^{65}Zn within the 24-hour period.

Since interference by cadmium with uptake of 3 μg of ^{65}Zn did not occur, and in-

TABLE 2

Uptake of ^{65}Zn by tissues when a basal dose of 27 μg was fed with zero, 5, 10 or 50 molEq of Ca (percentage of dose [1] given)

Calcium added [2]	Uptake for 24-hour period		
	Blood [3]	Liver [3]	Tibiae [3]
molEq			
0	1.20	3.38	1.36
5	1.11	3.46	1.38
10	0.95	3.09	1.18
50	1.08	3.15	1.18

[1] Twenty-seven micrograms of ^{65}Zn (0.915 μCi/mg Zn) as $^{65}ZnCl_2$ in 0.5 N HCl. Nine chicks per level of calcium.
[2] As a water solution of $CaCl_2$.
[3] Differences due to inclusion of calcium were nonsignificant at $P < 0.05$. Differences between each tissue for each time period were also nonsignificant.

TABLE 3

Uptake of ^{65}Zn by tissues when a basal dose of 3 μg was fed with zero, 5, 10 or 50 molEq of Cd (percentage of dose [1] given)

Cadmium [2] added	Uptake for 24-hour period		
	Blood [3]	Liver [3]	Tibiae [3]
molEq			
0	1.07	3.12	1.15
5	0.84	3.05	1.18
10	1.11	4.09	1.27
50	1.28	3.42	1.06

[1] As $^{65}ZnCl_2$ in 0.5 N HCl (0.915 μCi/mg Zn). Nine chicks per cadmium level.
[2] As a water solution of $CdCl_2 \cdot H_2O$.
[3] Differences between groups were nonsignificant at $P < 0.05$.

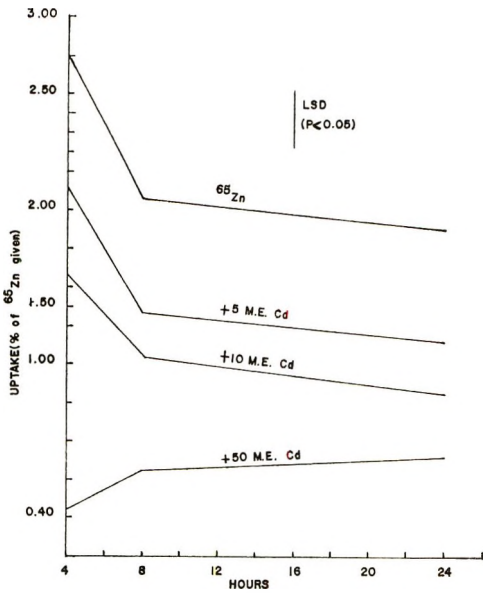

Fig. 1 Uptake of 18 μg of ^{65}Zn by the blood at 4, 8 and 24 hours when zero, 5, 10 or 50 molEq of cadmium were included. (Three trials combined; trials 1 and 2, ^{65}Zn (1.35 μCi/mg), diluted with deionized water to supply 18 μg of ^{65}Zn per chick. Nine chicks per cadmium level for each trial. Trial 3, ^{65}Zn (4.15 μCi/mg), diluted with a water solution of $ZnCl_2$ to supply 18 μg of zinc per chick; capsule counts per minute adjusted to approximate trials 1 and 2; 15 chicks/cadmium level.)

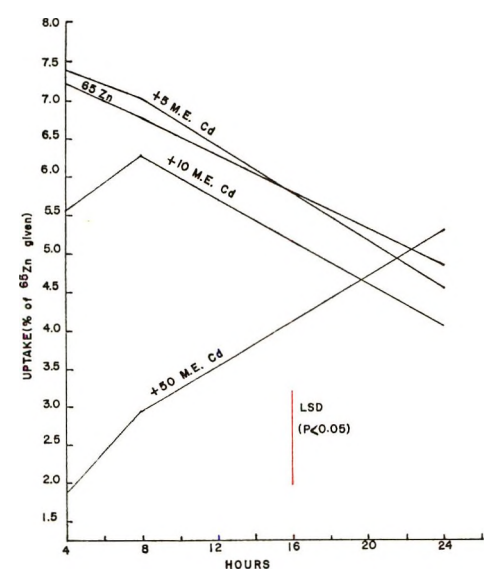

Fig. 2 Uptake of 18 μg of ^{65}Zn by the liver at 4, 8 and 24 hours when zero, 5, 10 or 50 molEq of cadmium were included. (See legend to fig. 1.)

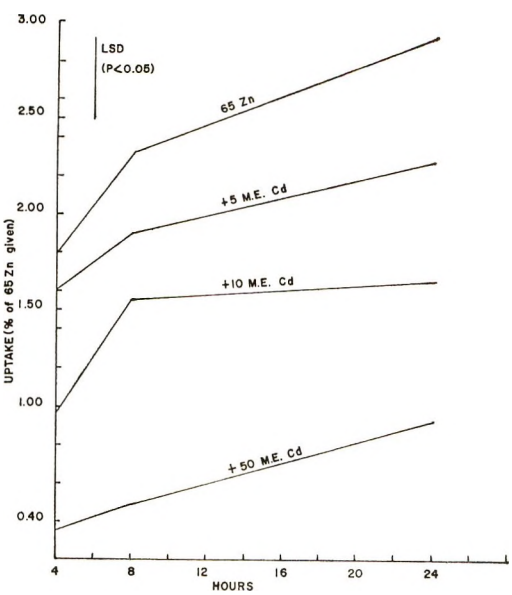

Fig. 3 Uptake of 18 μg of ^{65}Zn by the tibiae at 4, 8 and 24 hours when zero, 5, 10 or 50 molEq of cadmium were included. (See legend to fig. 1.)

terference by 5 and 10 molEq of cadmium were about the same with the basal dosage of 27 μg of ^{65}Zn (table 1), an intermediate dosage of 18 μg of ^{65}Zn was tried. The objective was to find a level of ^{65}Zn at which graded additions of cadmium would cause graded differences in absorption of ^{65}Zn and to follow uptake of the liver and tibiae under such circumstances. A graded, significant decrease in blood uptake of 18 μg of ^{65}Zn was found at each level of added cadmium (fig. 1) and differences in uptake by liver and bone occurred. Though zinc metabolism is governed by a homeostatic mechanism (7), cadmium is not (10). A large deposition of cadmium in any of the three tissues might disrupt this mechanism and cause excretion of absorbed ^{65}Zn through lack of deposition sites for ^{65}Zn. The uptake of ^{115m}Cd in the presence of 18 μg of stable zinc was measured to determine if some of the variations in uptake of ^{65}Zn over time could be due to an inverse relationship between cadmium and zinc uptake by the tissues.

The blood level of ^{115m}Cd was too low to be read. Little variation in the uptake of ^{115m}Cd by the liver (fig. 4) or the tibiae (fig. 5) over the 24-hour period for either 5 or 10 molEq of ^{115m}Cd occurred. Inclusion of

stable zinc had little effect on the uptake from the 5 molEq of ^{115m}Cd dosage. Apparently, variation in ^{65}Zn uptake with these equivalents of cadmium would not be due primarily to inverse variation in cadmium content of the tissues with time.

Large variations in the uptake of ^{115m}Cd by the liver or tibiae from 50 molEq of ^{115m}Cd occurred over the 24-hour period (figs. 4 and 5). The very high content of tibiae or liver at 8 hours, followed by a sharp decline by 24 hours, suggested that a detoxication reaction had taken place. Between 8 and 24 hours when the high levels of ^{115m}Cd were being reduced, presumably by excretion, the comparable ^{65}Zn content of the liver or tibiae increased significantly (figs. 2 and 3). Although it is a subjective observation, the chicks given 50 molEq of Cd with 18 μg or 27 μg of ^{65}Zn were very inactive and droopy compared with the other groups in all of the tests. In view of the nonphysiological uptake of cadmium and the physical condition of the chicks, the results with the 50 molEq of cadmium dosage are not included in the following discussion of the interactions of cadmium and zinc in relation to amount and pattern of uptake, and their application to dietary conditions.

Since the blood uptake from 18 μg of ^{65}Zn showed a graded decrease with increasing amounts of cadmium, suggesting that blood transport sites might be involved, and a varying inverse relationship between cadmium and zinc uptake with 5 or 10 molEq of cadmium apparently did not exist, the amount and pattern of uptake for the three tissues are given (figs. 1, 2 and 3) and discussed in detail. Much of the discussion can also be applied to the 27 μg of ^{65}Zn dosage (table 1), considering that the general pattern of uptake was similar but the amount of uptake varied little between the 5 and 10 molEq of Cd additions.

Uptake of 18 μg of ^{65}Zn over time (figs. 1, 2 and 3; $P < 0.05$)

At 4 hours. A significant decrease in uptake of ^{65}Zn by the blood was found at each level of added cadmium. The graded decrease suggests that there was competition between cadmium and zinc for binding sites in the blood as absorption occurred, with the result that the intake of ^{65}Zn was limited by the amount of cadmium present. Although competition for binding sites of the intestinal mucosa could give a similar graded decrease, the continued graded difference in blood uptake between levels of added cadmium over the 24-hour period suggests that cadmium did not act primarily to slow the passage of ^{65}Zn through the mucosa, but prevented absorption by occupying binding sites in the blood normally used by zinc.

In contrast to the decrease in blood uptake, the uptake by the liver was not significantly decreased by the presence of 5 molEq of cadmium. The usual pattern of ^{65}Zn uptake by the liver at 4 hours when absorption is low is a lesser uptake by the liver (8). The break in the pattern suggests that as early as 4 hours after ingestion, ^{65}Zn was accumulating in the liver when cadmium was also present. Furthermore, the ^{65}Zn uptake of the combined tissues of the basal chicks and those given 5 molEq of cadmium differed little (table 4); cadmium was not increasing the excretion of absorbed ^{65}Zn. For the chicks given 10 molEq of cadmium, however, the combined tissue content of ^{65}Zn was less; this is consistent with a lower absorption which also resulted in a significantly lower liver uptake. The failure of twice as much cadmium to increase liver stores of ^{65}Zn at this

TABLE 4

Combined tissue content of chicks given 18 μg of ^{65}Zn with zero, 5, 10 or 50 molEq of cadmium (percentage of ^{65}Zn given)

Time	0 Cd	5 molEq Cd	10 molEq Cd	50 molEq Cd
hour				
4	11.78	11.17	8.26	2.71
8	11.11	10.45	9.10	4.10
24	9.69	8.15	6.79	6.97

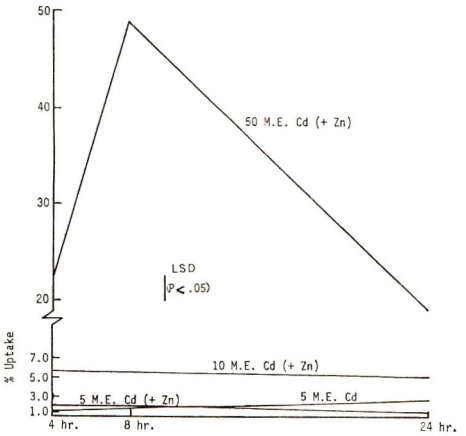

Fig. 4 Uptake of orally administered ^{115m}Cd (117 μCi/mg) by the liver at 4, 8 and 24 hours when given as 5, 10 or 50 molEq of 18 μg of zinc. One group given 5 molEq of ^{115m}Cd without added zinc. The other groups given 5, 10 or 50 molEq of ^{115m}Cd or ^{115m}Cd + stable cadmium, plus 18 μg of zinc. (Due to limitation of capsule size, it was necessary to include 5 molEq and 45 molEq of stable cadmium as $CdCl_2 \cdot 2H_2O$ to make up the 10 molEq and 50 molEq levels respectively. The actual activity measured for all groups was that of 5 molEq of ^{115m}Cd. The inclusion of the stable cadmium did not lower tissue activity; thus, the activity of the tissues of the chicks receiving 10 molEq and 50 molEq of Cd + ^{115m}Cd was multiplied by 2 and 10, respectively.)

time suggests that the presence of relatively large amounts of cadmium (fig. 4) did not in itself immediately cause retention of large amounts of zinc by the liver.

A significant difference in uptake of the tibiae did not appear between the chicks given the basal ^{65}Zn dosage and those given 5 molEq of cadmium; inclusion of 10 molEq of cadmium resulted in a significant decrease in uptake by the tibiae. The uptake of ^{65}Zn by the tibiae is relatively low at 4 hours; although the blood of chicks receiving 5 molEq of cadmium contained less ^{65}Zn than that of the basal ^{65}Zn chicks, apparently sufficient was present to satisfy the needs for uptake at this time and there was little interference with the mechanism governing the uptake of ^{65}Zn by the tibiae. The significantly lower uptake by the tibiae when 10 molEq of cadmium was included could reflect the lower concentration of ^{65}Zn in the blood and liver so that sufficient ^{65}Zn was not available for full uptake by the tibiae.

In summary, the observations at 4 hours indicated that increasing amounts of cadmium increasingly interfere with the absorption of ^{65}Zn, probably through competition for binding sites in the blood.

At 8 hours. The pattern of a decrease in blood level of ^{65}Zn shown by the basal ^{65}Zn group was followed by the 5 or 10 molEq of cadmium groups at a significantly lower level.

The break in the pattern of lower liver uptake after lower absorption continued for the chicks given 5 molEq of cadmium and was extended to the 10 molEq group. Despite a significantly lower blood level and a lower combined tissue content of ^{65}Zn (table 4), ^{65}Zn accumulated in the liver of the 10 molEq of cadmium group by 8 hours, so that differences among the three groups did not appear. With time, despite the lower level of ^{65}Zn in the blood, when cadmium was present the liver apparently had the capacity to take up enough ^{65}Zn from the circulating blood to reach the level obtained with the basal ^{65}Zn group. Whether the blood sites were replenished by further absorption of ^{65}Zn from the intestine, or uptake from other tissues not measured, is not known. When blood uptake was low due to lack of absorption of phytate-bound ^{65}Zn (8), a similar accumulation of ^{65}Zn in the liver did not occur.

The three groups followed the general pattern of increase in ^{65}Zn uptake by the tibiae by 8 hours. Even though the amount of uptake by the chicks given 10 molEq of cadmium was significantly lower than that of the basal group, the rate of increase suggests that the mechanism governing uptake of ^{65}Zn by the tibiae was little affected by cadmium at this time. Although the blood concentration of ^{65}Zn was low, such ^{65}Zn as was present could be taken up by the tibiae. The sharp rise in content of the tibiae suggests that the blood supply was being speedily replenished from the high level of ^{65}Zn in the liver.

A decrease in combined tissue content of ^{65}Zn of the chicks given 10 molEq of cadmium (table 4) did not occur by 8 hours. The presence of 10 molEq of cadmium, though limiting the blood level of ^{65}Zn and the total uptake of ^{65}Zn by the

tibiae, did not result in excretion of absorbed ^{65}Zn.

In summary, by 8 hours the pattern of uptake of ^{65}Zn by the blood of the three groups was similar, but the blood of the chicks fed cadmium contained significantly less ^{65}Zn than the controls. The level of ^{65}Zn in the liver did not differ significantly among the groups. Tibiae uptake was increasing for all groups but the rate of uptake by the 5 molEq chicks had slowed and the total uptake of the 10 molEq of cadmium group was significantly lower than that of the basal group.

At 24 hours. For the three groups, the pattern and amount of uptake of ^{65}Zn by the blood resembled those at 8 hours. The amount of liver stores did not vary significantly among the three groups and all followed the pattern of a significant decrease from 8 to 24 hours. A difference in the pattern and amount of uptake of ^{65}Zn by the tibiae, however, and differences in the amount of ^{65}Zn in the combined tissues (table 4), suggest that the destination of the decreased liver stores of ^{65}Zn varied.

At each time increment, the basal ^{65}Zn group had shown a significant increase in uptake by the tibiae. The chicks given 5 molEq of cadmium did not show a similar increase; by 24 hours a significant difference in amount of uptake between the two groups appeared. Tibiae uptake of the 10 molEq of cadmium group had essentially stopped between 8 and 24 hours and was significantly lower than that of the other groups. Despite a similar liver content of ^{65}Zn of the three groups, and a blood content comparable to that at 8 hours, the combined tissue of the chicks given 5 to 10 molEq of cadmium decreased more than that of the basal group. Since the blood content of ^{65}Zn differed little from that at 8 hours when uptake by tibiae occurred and liver stores were as high as those of the basal group, it is thought that the increased excretion was due to interference with uptake of ^{65}Zn by the tibiae. The intervention of some factor which slowed or prevented uptake by the tibiae in the presence of cadmium could result in excretion of some absorbed ^{65}Zn, which in the basal group was taken up by the tibiae.

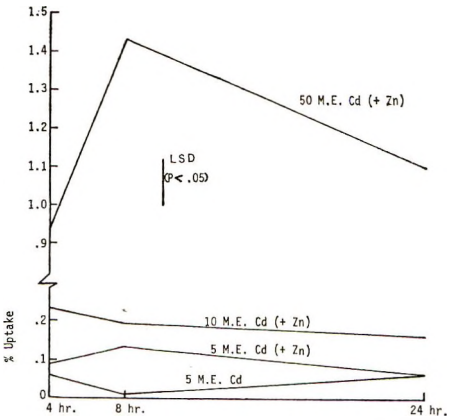

Fig. 5 Uptake of orally administered ^{115m}Cd by the tibiae at 4, 8 and 24 hours when given as 5, 10 or 50 molEq of 18 μg of zinc. One group given 5 molEq of ^{115m}Cd without added zinc. The other groups given 5, 10 or 50 molEq of ^{115m}Cd or ^{115m}Cd + stable cadmium, plus 18 μg of zinc. (See legend to fig. 4.)

Several explanations may be offered for the break in the pattern of uptake by tibiae between 8 and 24 hours in the presence of cadmium. The amount of uptake of ^{115m}Cd by the tibiae over the 24-hour period was fairly constant (fig. 5). If cadmium were occupying tibia sites ordinarily used by zinc, the cessation of uptake of ^{65}Zn by the 10 molEq of cadmium group between 8 and 24 hours could be due to lack of further sites for ^{65}Zn uptake. It would be expected, however, that the tibiae of the group given 5 molEq of cadmium would not show a slowing of uptake, but a sudden stoppage also, when the maximum capacity for uptake of the tibae was reached.

Since uptake of ^{65}Zn by the liver was high in the presence of cadmium, it seemed possible that cadmium occupied some of the sites ordinarily used by zinc. The ^{65}Zn then might be bound by other sites from which it was not readily available for transport by the blood to the tibiae. That the liver can store zinc in sites not usually used is shown by the storage of zinc in sites normally occupied by iron when excess zinc was fed (11). The binding of zinc by "abnormal" sites could account for the failure of a liver level of ^{65}Zn comparable to the basal ^{65}Zn group to furnish enough ^{65}Zn to produce comparable uptake by the tibiae. When calves were fed 640

ppm of cadmium, blood levels of zinc were lower than those of the controls and the liver content was higher (1). The calves did not use the liver stores to prevent the appearance of parakeratosis. In the chick, a low content of zinc in the tibiae is one of the symptoms of a zinc deficiency. The association of higher liver levels of zinc with signs of zinc deficiency in the calf, and with the lowered tibia content in this experiment, lend support to the hypothesis that the liver stores of ^{65}Zn were little available to the animal.

Since the pattern of tibia uptake was altered, the possibility of interference by cadmium with the action of alkaline phosphatase was considered.[4] The suggestion has been made that the zinc content of bone increases as new calcification occurs (12). If the presence of cadmium reduced alkaline phosphatase activity, and thus ossification and concomitant uptake of ^{65}Zn by the bone, a lower rate of uptake by the tibiae, dependent on the amount of cadmium present and the amount of ^{65}Zn available to be incorporated in the bone, would be expected. The higher level of cadmium furnished by the 10 molEq dosage would be expected to have a greater effect than that of the 5 molEq dosage on tibia uptake.

The slowing in rate of uptake of ^{65}Zn in the presence of 5 molEq of cadmium and cessation of uptake when 10 molEq of cadmium was included may be due to a combination of these possibilities or to some other factor.

In summary, by 24 hours the blood content of ^{65}Zn was comparable with that at 8 hours, graded differences due to inclusion of cadmium were still present. The pattern of uptake was similar for the three groups. The uptake of the liver of the cadmium groups did not vary in amount or pattern from the basal ^{65}Zn group. The destination of the decreasing stores of liver-^{65}Zn differed, however; in the basal group, tibia uptake was increased with some excretion; in the groups fed cadmium, the rate of tibia uptake was decreased or had ceased and excretion of absorbed zinc was greater.

Although it will be necessary to isolate a blood-binding substance which is used in the transport of both zinc and cadmium, the amount and pattern of uptake in the present experiments suggest that such a substance exists. The early graded decrease in amount of ^{65}Zn uptake found when 5 and 10 molEq of cadmium were fed persisted over the 24-hour period. The pattern of decrease in blood uptake over time shown by the basal ^{65}Zn group was followed by the 5 and 10 molEq of cadmium groups. This would be consistent with a homeostatic mechanism regulating the blood content of ^{65}Zn, regardless of the absolute amount of ^{65}Zn present, and is similar to the pattern found when absorption of ^{65}Zn is limited (8). Since the combined tissue content of the basal and 5 molEq of cadmium groups was comparable at 4 and 8 hours, whereas that of the 10 mol Eq of cadmium chicks increased by 8 hours, the decrease in blood uptake does not seem to be due to an increased excretion of absorbed ^{65}Zn caused by cadmium. The increased loss of absorbed ^{65}Zn by the combined tissues seen at 24 hours in the birds fed cadmium, without a corresponding rise in blood level of ^{65}Zn, would be consistent with a situation in which some of the binding sites of the blood were occupied by cadmium so that more absorbed zinc could not be transported to the site of excretion at any one time. Instead, the same sites could be used repeatedly as circulation continued. When calves were given a diet containing cadmium, subsequent uptake by the blood of a single dose of ^{65}Zn was lower than that of the controls (13). This would be consistent with occupation of some blood-binding sites by the cadmium previously fed.

Although the results presented here only measured the zinc and cadmium content of some tissues, retention of zinc by the liver, which is also found in comparatively long-time dietary studies, suggests that not only is the ^{65}Zn taken up by the liver little available to the chick, but that the presence of large quantities of zinc in the liver may interfere with metabolism of other minerals. Even though the simultaneous administration of cadmium decreases absorption of zinc, the accumulation of zinc in the liver in the presence of

[4] Britton, W. M., and C. H. Hill 1967 The influence of cadmium and copper on zinc deficiency. Federation Proc., 26: 523 (abstract).

cadmium may have the same effect as high dietary zinc as far as liver metabolism is concerned.

In applying the effects of cadmium on the uptake of ^{65}Zn to dietary interrelationships of cadmium and zinc, apparently both the level of zinc and cadmium fed, and the proportion of one to the other are involved. At low dietary levels of cadmium and zinc, interference may not occur. Cadmium is retained in the liver and tibiae (figs. 4 and 5), however, and apparently occupies zinc-binding sites in the blood. Over a relatively long period of time, with ingestion of even small amounts of cadmium, owing to lack of homeostatic control (10), the body could accumulate enough cadmium to interfere with absorption of zinc by means of increasing occupation of blood-binding sites.

The comparable depression in uptake of ^{65}Zn by the blood or tibiae caused by the inclusion of 5 or 10 molEq of Cd with 27 μg of ^{65}Zn, in contrast to the graded decreases with increasing levels of cadmium which appeared when 18 μg of ^{65}Zn was given, suggests that as the absolute amount of cadmium fed is increased, the proportion of cadmium taken up increases. Cadmium can interfere with the absorption of zinc and cause disturbances at a more fundamental level, but the magnitude of these interferences apparently cannot be predicted on a proportional basis; the basal level of zinc to which cadmium is related can make a difference. Since cadmium is not subject to homeostatic control, the length of the experimental feeding period also probably will have an effect. Similarly, since the number of transport sites in the blood will vary with the size of the blood pool, the size of the experimental animal may be a factor in cadmium and zinc interrelationships. All these factors should be taken into consideration when discussing cadmium and zinc interrelationships.

ACKNOWLEDGMENTS

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Effect of Amino Acid Imbalance on Plasma and Tissue Free Amino Acids in the Rat [1]

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ABSTRACT Plasma and tissue free amino acids were measured in rats trained to eat a single meal for 1 hour daily, at intervals after they had been fed a low protein diet or an amino acid-imbalanced diet containing 5.4% of an amino acid mixture lacking threonine. The plasma concentration of the most limiting amino acid, threonine, in rats fed the imbalanced diet, fell rapidly below both the fasting value and the value for rats fed the control diet. The pattern of changes of threonine in the systemic plasma appeared to be a reflection of the changes occurring in the muscle. The pattern of changes in the liver, however, differed from those in muscle and plasma. Threonine concentration in liver of rats fed the imbalanced diet did not fall below that of the control at the 3- and 5-hour intervals. Threonine concentration of intestinal tissues of the imbalanced group was high compared with those of plasma and muscle. Plasma threonine concentration of rats fed the imbalanced diet ad libitum paralleled food intake rather closely, i.e., threonine concentration fell below that of the control when food intake was depressed, but at the end of the 22-day experimental period increased above the control value when the food intake also increased above that of the control. Plasma amino acid pattern of rats fed a protein-free diet more closely resembled that of rats fed the control diet.

The most consistent biochemical change that occurs within a short time in rats fed an imbalanced diet is a change in plasma amino acid pattern. In particular, the plasma concentration of the most limiting amino acid falls and this occurs prior to or concurrently with a depression in food intake (1, 2). It has been suggested that the altered plasma amino acid pattern may directly or indirectly affect the food intake regulatory mechanism (3).

The plasma amino acid patterns of several tissues of rats trained to eat their daily food in a single 1-hour period and fed an imbalanced diet containing a low level of casein and a mixture of indispensable amino acids lacking threonine have been examined in the present study. The plasma concentrations of free amino acids have been compared with those of skeletal muscle, liver, intestine and intestinal contents. The plasma amino acid patterns of rats fed an imbalanced diet ad libitum for 1, 3, 7 and 22 days were also determined in an attempt to follow the changes of the plasma amino acid pattern during periods in which changes in food intake occur. The plasma amino acid pattern of rats ingesting a protein-free diet and pair-fed to the animals fed the imbalanced diet was also examined.

EXPERIMENTAL

Male rats of the Holtzman strain were used in all the experiments. The basal diet used was a 6% casein diet supplemented with 0.3% DL-methionine. The diet contained: (in percent) casein, 6; salt mixture,[2] 4; corn oil,[3] 5; vitamin mixture,[4] 0.5; choline chloride, 0.2; and dextrin–starch (1:2) to make up 100%. The imbalanced

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[2] The salt mixture contained: (in percent) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; Fe $(C_6H_5O_7) \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.0025; and $Na_2SeO_3 \cdot 5H_2O$, 0.0015. The salt mix was prepared by and purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. The salts at 5% of the diet provided in percent of element: Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; and Se, 0.0000025.

[3] Mazola, Corn Products Company, New York.

[4] 0.5% of the vitamin mixture in the diet provided the rats with 0.44% sucrose plus the following vitamins: (in mg/kg diet) thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% in mannitol), 30; ascorbic acid, 50 (added to prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of vitamin A/g and 32,500 USP units of vitamin D_2/g in a mixture of gelatin, sugar and starch), 12.31. (The thiamine, niacinamide, folic acid, menadione and vitamin B_{12} were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and the rest were purchased from Hoffmann-La Roche, Inc., Nutley, New Jersey.)

diet was prepared by adding 5.4% of an amino acid mixture devoid of threonine [5] to the basal diet. The addition of amino acids was compensated for by adjusting the percentage of the mixed carbohydrate.

The rats (100 to 120 g) used in the single-meal study were trained to consume their daily ration in 1 hour. They were fed during the 4-week training period a 15% casein diet containing adequate quantities of all essential nutrients and a mixed carbohydrate (dextrin–starch, 1:2). Rats that gained the same amount of weight and were consuming approximately the same amount of food were separated into 2 groups. The imbalanced group was fed the imbalanced diet for 1 day. The control rats were pair-fed against the rats fed the imbalanced diet. The average food consumption of each group was 8 g. Samples of blood (by cardiac puncture), skeletal muscle (from the right hind leg), liver, and the entire small intestine and intestinal contents were taken and the samples from 5 rats were pooled at 1.5, 3, 5, 8, 13, 19 and 24 hours after feeding. Samples of blood and tissues were also taken from a group of 5 rats held without food for 24 hours.

In the study of plasma amino acids of animals fed ad libitum, 70-g rats were fed the basal or the imbalanced diet ad libitum for 22 days. Blood samples were withdrawn by cardiac puncture from 5 rats in each group on day zero, day 1, and day 3 and from 4 rats on day 7 and day 22. Another identical group of rats was pair-fed for 8 days an amount of protein-free diet equal to the amount of food ingested by the imbalanced group. The plasma amino acids of rats fed the protein-free diet were not determined after 22 days because these rats progressively reduced their food consumption after 8 days, whereas the imbalanced group showed a steady increase in food intake. Blood samples were withdrawn on days 1, 3 and 7 from the same numbers of animals pair-fed the protein-free diet.

All blood samples were treated by methods similar to those of Stein and Moore (4). Protein-free blood filtrates were prepared using 1% picric acid. Portions of liver and skeletal muscle (weighing 1 to 2 g) and the whole small intestine were quickly excised from the animals, weighed and cut into small pieces to which cold 1% picric acid solution was added in a 1:10 ratio (tissue to picric acid solution) according to the method of Hamilton (5). The small intestinal contents, prior to the above treatment, were rinsed from the small intestine with distilled water. Picric acid was added to the intestinal contents to give a final concentration of 1%. Samples were homogenized and centrifuged. The picric acid was then removed from the supernates by passing them through a column of Dowex 2-X8 resin (50 to 100 mesh, chloride form) as described by Tallan et al. (6). The picric acid-free supernates were then concentrated to a small volume in a flash evaporator. The final concentrate was made up to volume with 0.02 N HCl. Aliquots of the final solution were used for the determination of amino acids using a Technicon amino acid analyzer. Tryptophan was not determined by this procedure.

RESULTS

Single-meal feeding. The stomach-emptying patterns of both the control and imbalanced groups were determined and were found to be similar. By 13 hours after feeding, only small amounts (1 to 3%) of the ingested diet remained in the stomach.

Amino acid pattern of blood plasma. Changes in amino acid concentrations over a 24-hour period in the plasma of rats fed 8 g of either the control or the imbalanced diet are shown in figure 1. Within 3 hours the concentration of the most limiting amino acid, threonine, in the plasma of the group fed the imbalanced diet fell well below the fasting and the control values. The values for threonine in the plasma of the imbalanced group remained below those for the control until after the 13-hour period. By 19 hours the plasma threonine concentration of the imbalanced group was above that of the controls, at which time the stomachs of both groups were found to be empty. The biggest difference between the concentrations of

[5] The mixture of indispensable amino acids lacking threonine provided: (in percent of diet) DL-tryptophan, 0.24; L-leucine, 0.84; DL-isoleucine, 1.2; DL-valine, 1.30; L-histidine·HCl, 0.36; DL-phenylalanine, 0.54; and L-lysine·HCl, 0.90.

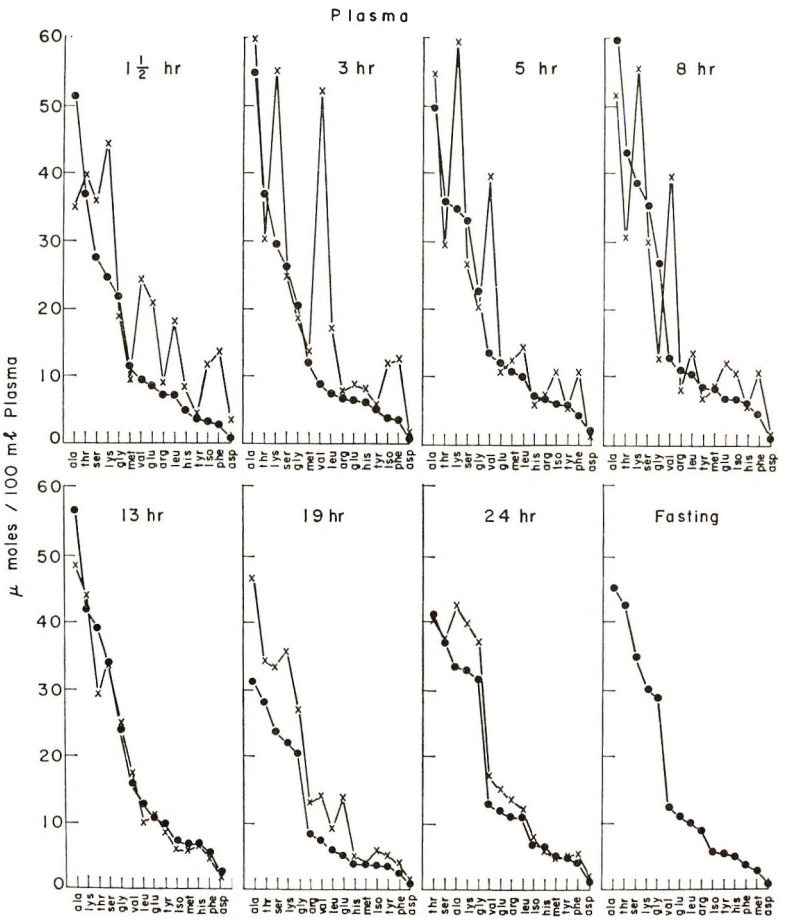

Fig. 1 Plasma aminograms of rats trained to eat a single 1-hour meal daily and fed the control diet or the imbalanced diet. ·———·, Control diet: 6% casein plus 0.3% DL-methionine; and x———x, imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

threonine in plasma from the control and the imbalanced group was observed 8 hours after the feeding period. The plasma concentrations of threonine for both the control and the imbalanced group were below the fasting concentration at all times. The methionine concentrations in the plasma were similar for both the control and the imbalanced groups with the imbalanced group having slightly higher values at 3 and 5 hours.

The concentrations of the other indispensable amino acids in the plasma of the groups fed the control diet had similar patterns throughout the 24-hour period. The values fell below the respective fasting values by 1.5 hours, then increased gradually and reached peaks above the fasting values 13 hours after the feeding period. This was followed by a drop to the lowest concentration at 19 hours, with a subsequent rise to approximately the fasting values at the end of the 24-hour period.

In plasma of rats fed the imbalanced diet, the patterns for phenylalanine, leucine and isoleucine, three of the indispensable amino acids which were added to cause the imbalance, were similar. The concentrations of these amino acids had increased sharply by 1.5 hours after the feeding period, remained elevated through the 8-hour period, but by 13 hours were

not different from the control values. The plasma concentration of valine did not reach a peak until 3 hours; the lowest value was observed at 19 hours. The concentration of lysine did not reach the maximum until 5 hours in rats fed the imbalanced diet. Plasma histidine in these rats behaved somewhat differently from the rest of the indispensable amino acids. There was a rise in histidine concentration within 1.5 hours but the values fell below those of the controls at 5 and 8 hours; thereafter, the pattern more closely resembled that for the controls.

The concentrations of plasma dispensable amino acids exhibited irregular patterns. Plasma concentrations of glutamic and aspartic acids were consistently higher in the imbalanced groups except at 5 hours. The values for tyrosine in the plasma of the imbalanced groups were similar to those for the control groups except at 8 and 13 hours when the concentrations were slightly lower in the plasma of the imbalanced groups.

Amino acid pattern of muscle. The changes in concentrations of free amino acids in the muscle at various time intervals throughout the 24-hour period are shown in figure 2. In contrast to the plasma amino acid pattern, some of the indispensable amino acids which were added to cause the imbalance did not increase as markedly above the control values as those

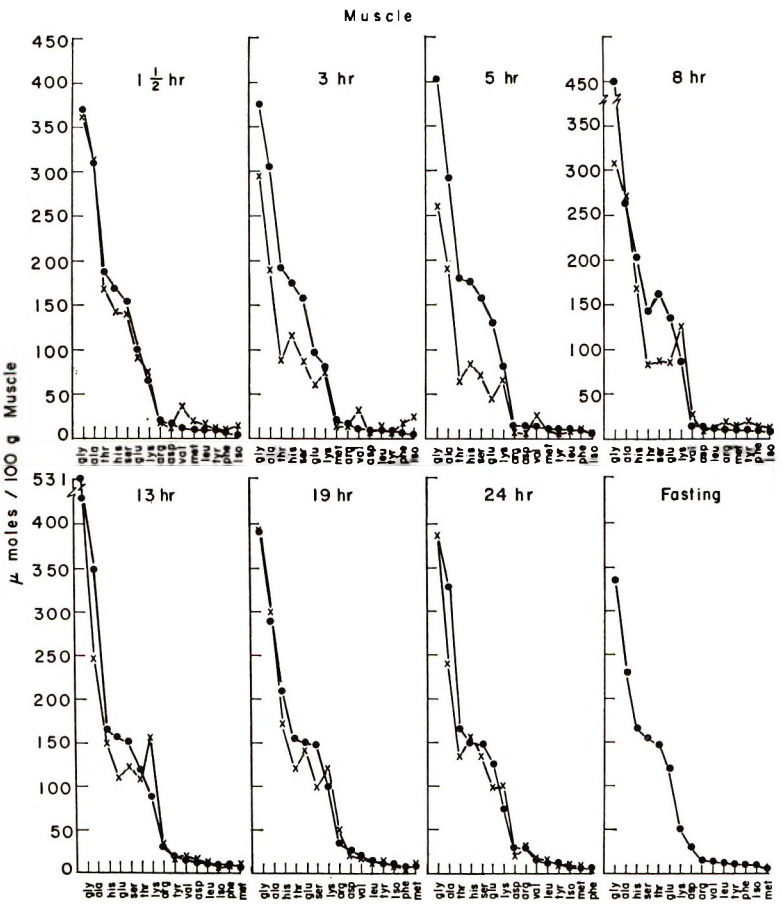

Fig. 2 Aminograms of muscle of rats trained to eat a single 1-hour meal daily and fed the control diet or the imbalanced diet. ·———·, Control diet: 6% casein plus 0.3% DL-methionine; and x———x, imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

in the plasma. All of the dispensable amino acids were lower in the muscle of rats fed the imbalanced diet. Pattern of changes in the threonine concentration in the muscle of the control and the imbalanced groups was similar to that for plasma. The maximum and the minimum concentrations of threonine, however, in the muscle of the control group occurred at 3 and 13 hours, respectively, whereas those for the plasma of the control groups occurred at 8 and 19 hours. The concentration of threonine in the muscle of the imbalanced group was markedly depressed by 3 hours. The lowest value was obtained at 5 hours; thereafter the values rose steadily towards the fasting level. The pattern of change in methionine concentration in the muscle for the control groups resembled that in plasma. The concentration of methionine in the muscle of the imbalanced group was higher than that of the control at 1.5 hours but somewhat lower at 3 and 5 hours. The values for phenylalanine, leucine, isoleucine and valine in the muscle of the control groups did not fluctuate as much as those for plasma; after a slight decline below the fasting value, 1.5 hours after the feeding period, they slowly returned to the fasting values at 5 hours and then remained essentially constant. The concentrations of these amino acids in the muscle of the imbalanced group also did not rise as markedly as in the plasma. This was especially true for leucine.

The pattern of change for lysine in the muscle of the imbalanced group was quite different from that in the plasma. The lysine concentration in the muscle did not increase above the control value until 8 hours after the feeding period. This was followed by a further rise to the highest point at 13 hours.

The changes in concentration of histidine in the muscle of the imbalanced group shortly after feeding resembled those for threonine, even though histidine was added to the imbalanced diet.

The concentrations of serine and glutamic acid in the muscle of the control group were higher than those in the imbalanced group at all times. The glycine concentration was higher in the control group in the early periods until after 8 hours. Alanine, aspartic acid, tyrosine and arginine showed no consistent patterns in the groups fed either of the two diets.

Amino acid pattern of liver. The relative changes in the concentrations of the amino acids in the livers of both the control and the imbalanced groups at various time intervals during the 24-hour period are shown in figure 3. The concentration of threonine in the liver of the imbalanced group fell rapidly below that of the control at 1.5 hours, whereas the concentrations of methionine, phenylalanine and histidine fell to a lesser degree. The concentrations of leucine and valine, however, were higher than the values of the controls at 1.5 hours, whereas the concentrations of lysine and isoleucine remained similar to those of the controls.

The concentrations of all the dispensable and indispensable amino acids were higher at 3 and 5 hours in the livers of rats fed the imbalanced diet than in those of the controls. The concentration of threonine at 5 hours was more than twice as high in the livers of rats ingesting the imbalanced diet, even though the intake of threonine was the same for both groups.

The concentrations of glutamic acid, alanine and glycine in the liver of the imbalanced group were considerably higher than the values for the controls, except at 1.5 and 24 hours after feeding. No regular patterns were observed for the concentrations of serine and aspartic acid in the livers of either groups. Tyrosine concentration was slightly lower in the liver of the control group except at the end of the 24-hour period. Arginine concentrations in the livers of both the control and the imbalanced groups were too low to permit accurate measurements and comparisons.

Amino acid pattern in intestinal tissue. Figure 4 shows the relative changes in the concentrations of the amino acids in intestinal tissues of the control and the imbalanced groups. The concentrations of all of the indispensable amino acids usually reached a peak at 5 or 8 hours in the intestinal tissues of both the control and the imbalanced groups, but the concentrations were higher in the imbalanced group at all times. The highest value for valine in the intestinal tissues of rats fed the imbalanced diet occurred at 8 hours.

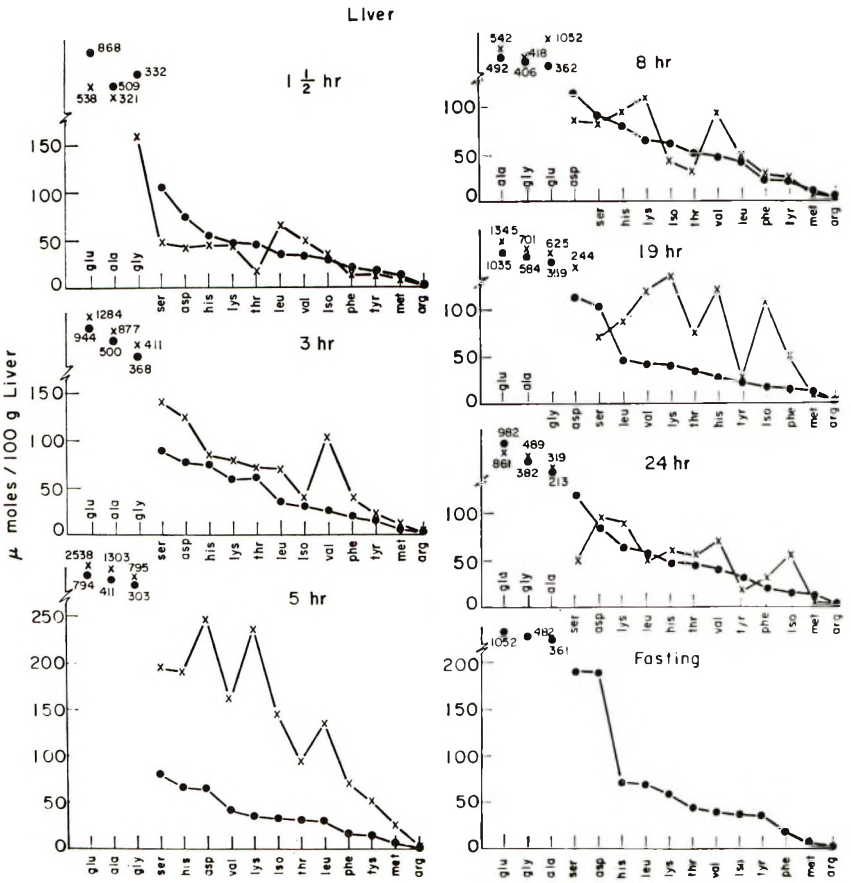

Fig. 3 Aminograms of liver of rats trained to eat a single 1-hour meal daily and fed the control or the imbalanced diet. ·———·, Control diet: 6% casein plus 0.3% DL-methionine; and x———x, imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

Except for alanine, arginine and tyrosine at 8 hours, the concentrations of all the indispensable and dispensable amino acids in the intestinal tissues of the imbalanced group were higher than in those of the control group at all times.

Amino acid patterns in intestinal contents. The amino acid patterns at various time intervals during the 24-hour period for the total amount of free amino acids found in the intestinal contents of rats fed the control or the imbalanced diet are shown in figure 5. The concentration of threonine in the intestinal contents of the control rats was rather constant at all times, whereas values for the imbalanced group were higher than those of the control group at 3 and 5 hours.

The amount of valine in the intestinal contents from the imbalanced group was much higher at 1.5, 3, 5 and 8 hours than the amounts of other indispensable amino acids added to cause the imbalance.

The levels of phenylalanine, leucine, isoleucine, lysine and histidine were higher in the contents from the imbalanced group at 1.5, 3 and 5 hours than in the contents from the corresponding controls. At 3 hours, the intestinal contents from the imbalanced group contained more of every amino acid than the contents from the control group, but by 8 hours, most of the amino acids were present in lower amounts in the contents from the imbalanced group than in those from the control; valine, however, was still elevated and phenyl-

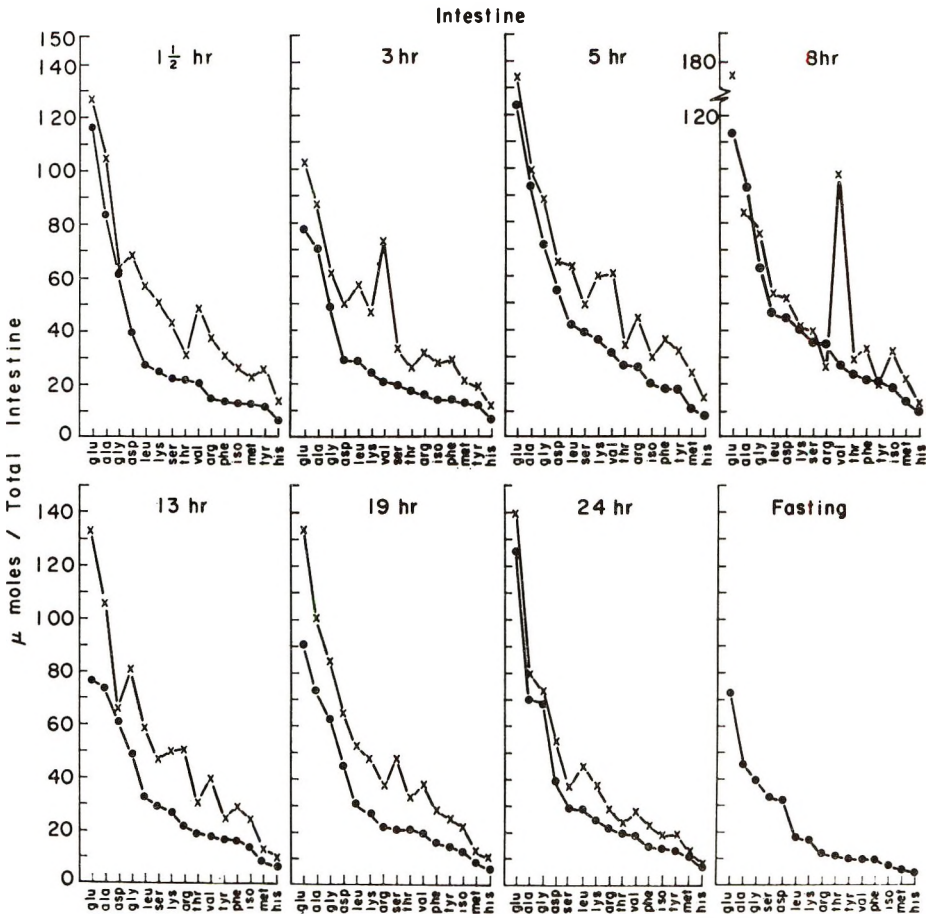

Fig. 4 Aminograms of intestinal tissues of rats trained to eat a single 1-hour meal daily and fed the control or the imbalanced diet. ·————·, Control diet: 6% casein plus 0.3% DL-methionine; and x————x, imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

alanine, isoleucine, threonine, methionine and histidine were present in about the same amounts in both groups.

The concentrations of all of the amino acids in the contents of both the control and the imbalanced groups were similar at 13 and 19 hours. At the end of the 24-hour period, however, the amounts of all the amino acids in the contents from the control group had increased, whereas those from the imbalanced group remained about the same. There was a marked elevation of all the amino acids in the intestinal contents of fasting animals.

Effect of amino acid imbalance on blood amino acid patterns of rats fed ad libitum. When rats were fed the imbalanced diet ad libitum, the usual food intake depression was observed. The rate of food intake of the imbalanced group after 20 days, however, was similar to or even slightly higher than that of the controls. Animals fed the protein-free diet lost weight progressively from the beginning of the experiment.

Plasma amino acid patterns of rats fed ad libitum for 1, 3, 7 and 22 days are shown in figures 6 and 7. There was a drop within 1 day in the plasma concentration of threonine in rats fed the imbalanced diet. The average food intakes of the control and imbalanced groups the day

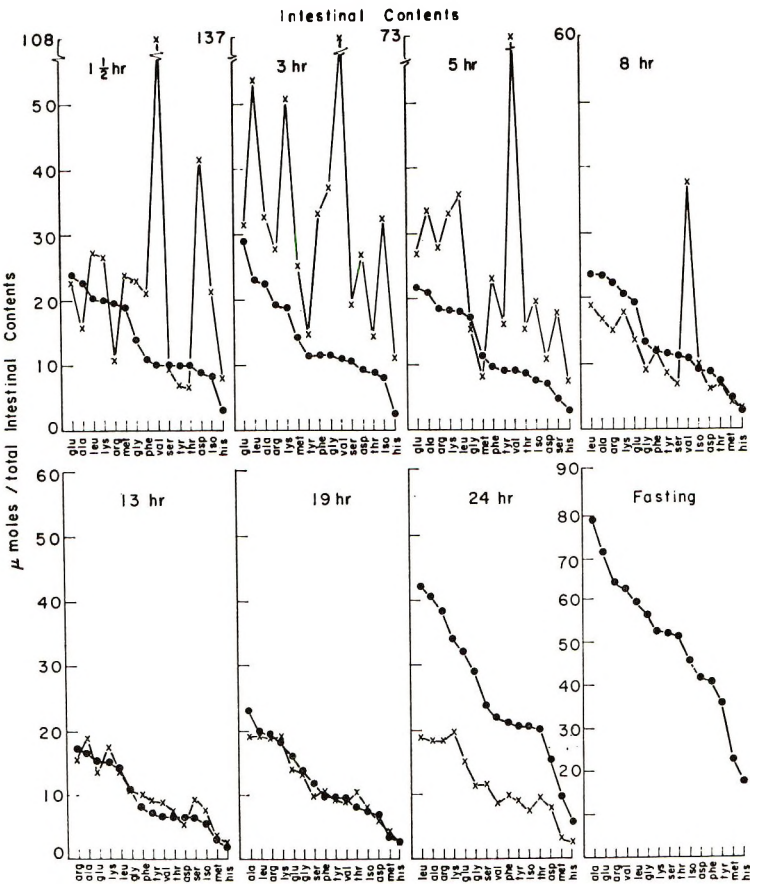

Fig. 5 Aminograms of intestinal contents of rats trained to eat a single 1-hour meal daily and fed the control or the imbalanced diet. ·———·, Control diet: 6% casein plus 0.3% DL-methionine; and x———x, imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

before they were killed were 7.0 and 2.4 g, respectively. The threonine concentrations in the plasma of the imbalanced group did not drop any more on day 3 even though the average food intake of the control and imbalanced groups the day prior to taking the blood samples was 6.4 and 2.8 g. The threonine concentration fell somewhat lower on day 8, even though the food intake had increased to 10.0 and 5.5 g, respectively. On day 22, however, the threonine concentration in the plasma of rats ingesting the imbalanced diet was higher than the control value and the average food intake of the control and the imbalanced groups was 9.1 and 14.2 g, respectively.

The plasma threonine concentrations of the groups fed the basal diet declined steadily during the experimental period; but each value was consistently higher than the comparable value for the imbalanced groups except on day 22, when the food intake of the imbalanced group was no longer depressed.

The methionine concentrations in the plasma of the imbalanced group were depressed markedly all through the experimental period.

The plasma concentrations of phenylalanine, leucine, isoleucine in rats fed the imbalanced diet were slightly lower than the control values during the first day, this was followed by a sharp rise above

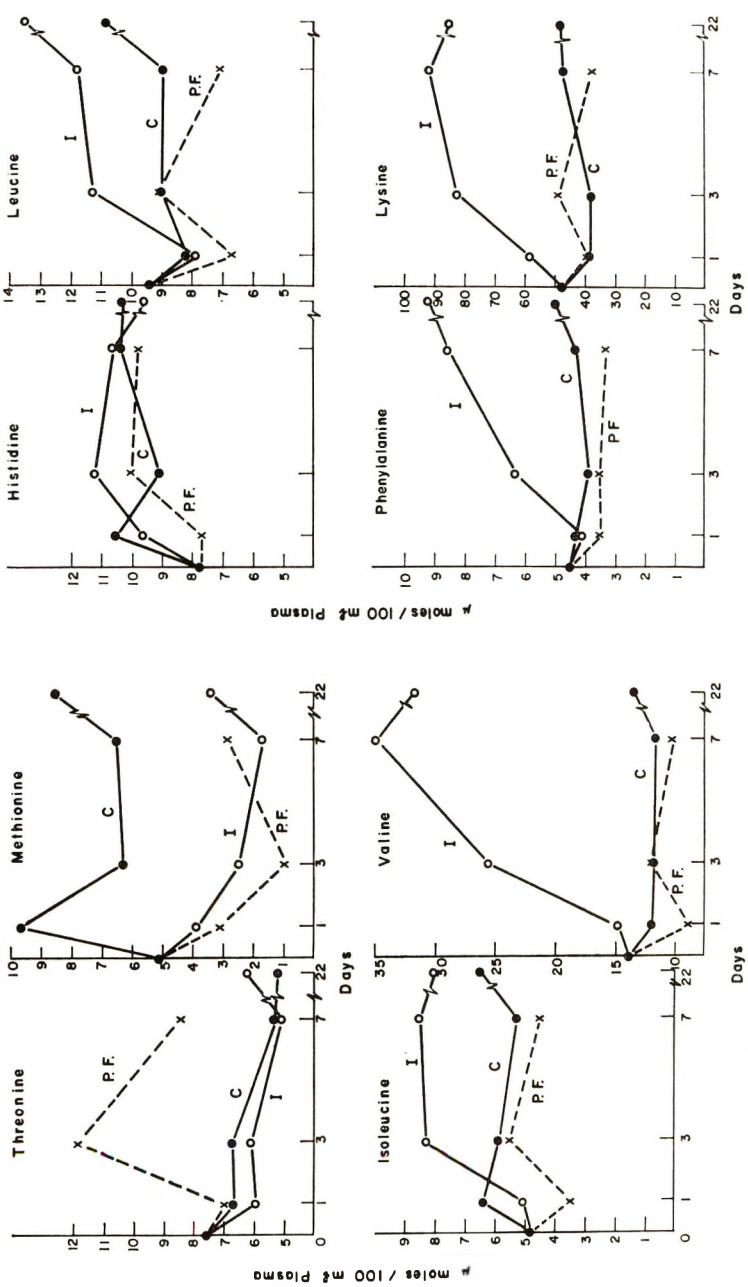

Fig. 6 Effect of amino acid imbalance on the indispensable amino acid concentrations in plasma of rats fed ad libitum the control diet (C), the imbalanced diet (I) and the protein-free diet (PF). Control diet: 6% casein plus 0.3% DL-methionine; imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; and protein-free diet: same composition as the control diet except that protein and methionine were replaced by mixed carbohydrate (dextrin–starch, 1:2).

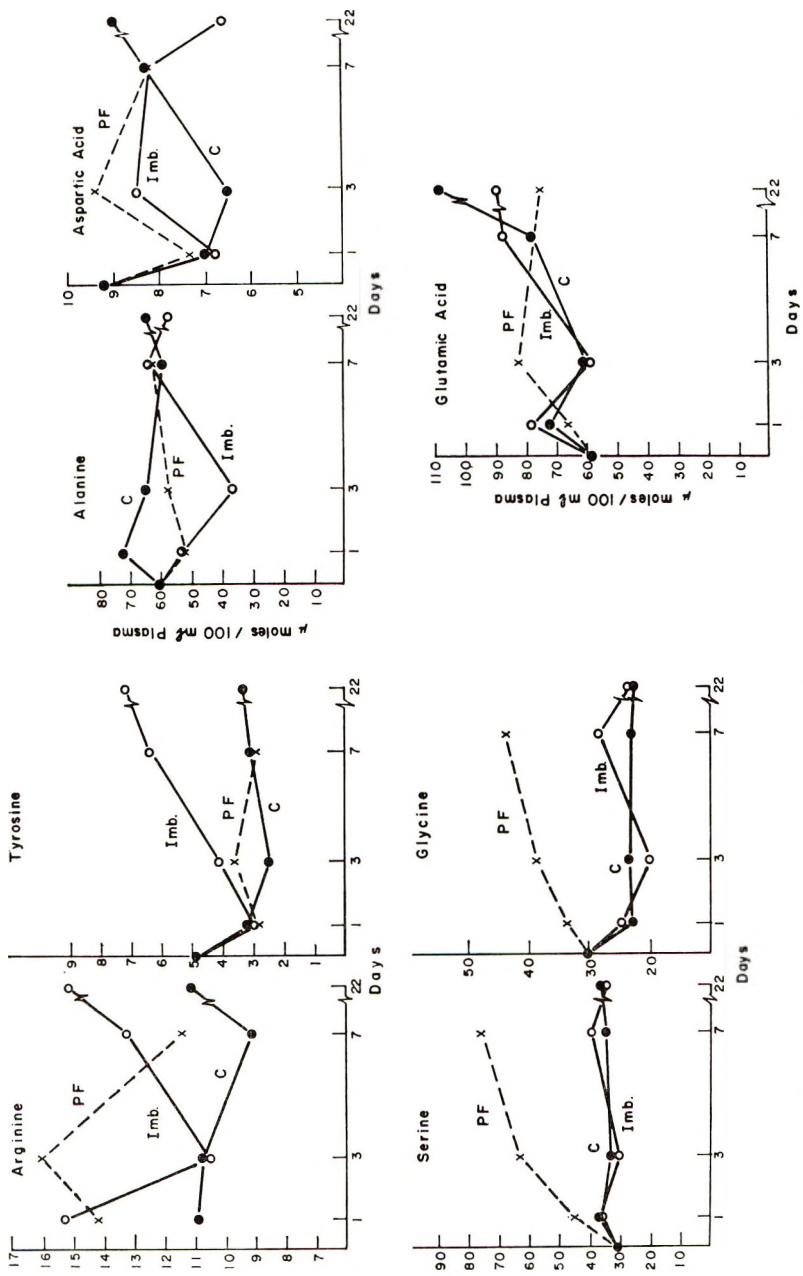

Fig. 7 Effect of amino acid imbalance on dispensable amino acid concentrations in plasma of rats fed ad libitum the control diet (C), the imbalanced diet (Imb), and the protein-free diet (PF). Control diet: 6% casein plus 0.3% DL-methionine; imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine; and protein-free diet: same composition as the control diet except that the protein and methionine were replaced by mixed carbohydrate (dextrin–starch, 1:2).

the control values on day 7 with the highest value being reached on day 22.

The concentrations of valine and lysine in the plasma of the imbalanced group, however, increased markedly above the control from the start and reached a peak on day 7. The plasma histidine concentration in rats fed the imbalanced diet, however, was not increased over that of the controls as markedly as those of the other indispensable amino acids added in the imbalanced diet. The histidine value was slightly lower than that of the control during the first day and increased only slightly over the control value on day 3 and remained similar to the control value from day 7 to the end of the experiment. The plasma indispensable amino acid concentrations, except for methionine and histidine of the group fed the basal diet, remained high during the 22-day experiment.

There was a marked rise within 3 days in the plasma concentration of threonine in rats fed the protein-free diet. Although the threonine concentration had decreased substantially by day 7, the value was still considerably higher than those for the other two groups.

The concentrations of phenylalanine, leucine, isoleucine, valine, histidine and lysine in the plasma of the group fed the protein-free diet were close to or slightly lower than the respective control values during the first day. The values remained either the same or slightly higher than the respective controls on day 3, and relatively unchanged or slightly lower on day 7. The blood concentrations of methionine, however, followed a pattern similar to that of the imbalanced group and were much lower than the controls at all time intervals.

The changes in pattern of serine, glycine and glutamic acid concentrations for both the control and imbalanced groups, were similar during the 22-day experiment. Whereas the plasma concentrations of serine and glycine in rats fed the protein-free diet were much elevated, the glutamic acid concentration for the group fed the protein-free diet was somewhat increased by 3 days and remained higher than the starting value throughout the experimental period. Plasma tyrosine concentration of the imbalanced group increased steadily after the first day, whereas the values for the control and the protein-free groups remained relatively constant throughout. The changes in the concentrations of alanine, aspartic acid, and arginine were quite irregular for groups fed the control, the imbalanced or the protein-free diet.

DISCUSSION

Amino acid imbalances are known to affect amino acid metabolism in some way. There appears to be a common pattern in all amino acid imbalances in that the plasma concentration of the most limiting amino acid is reduced below that of respective control values. Also, the concentrations of the amino acids added to cause the imbalance are normally markedly increased (1, 2, 7).

In the present study, the concentration of threonine, the most limiting amino acid, decreased in the plasma of rats fed the imbalanced diet and although the decrease was not as marked as that reported for histidine imbalance (1), the trend was the same. To illustrate more clearly the relationship of time-after-feeding, with the rise and fall of threonine in the blood and the various tissues, we have replotted the data as micromoles of the threonine per unit of tissue versus time (fig. 8). The concentration of plasma threonine had fallen by 3 hours after the period of feeding and remained lower than that of the controls for the entire absorption period. Plasma threonine concentration of the imbalanced group was not depressed by 1.5 hours. This may be because the animals were trained to eat a single meal of a 15% casein diet daily, instead of the low protein control diet. Also, the fasting level of threonine in the intestinal contents was high, thus endogenous threonine in the intestinal lumen may influence plasma threonine concentration immediately after food is eaten.

The fall of the limiting amino acid in the plasma of rats ingesting the imbalanced diet was not a result of delayed stomach emptying as evidenced from observations in this study and that of Kumta and Harper (1). In the present study, the stomach-emptying patterns for both the

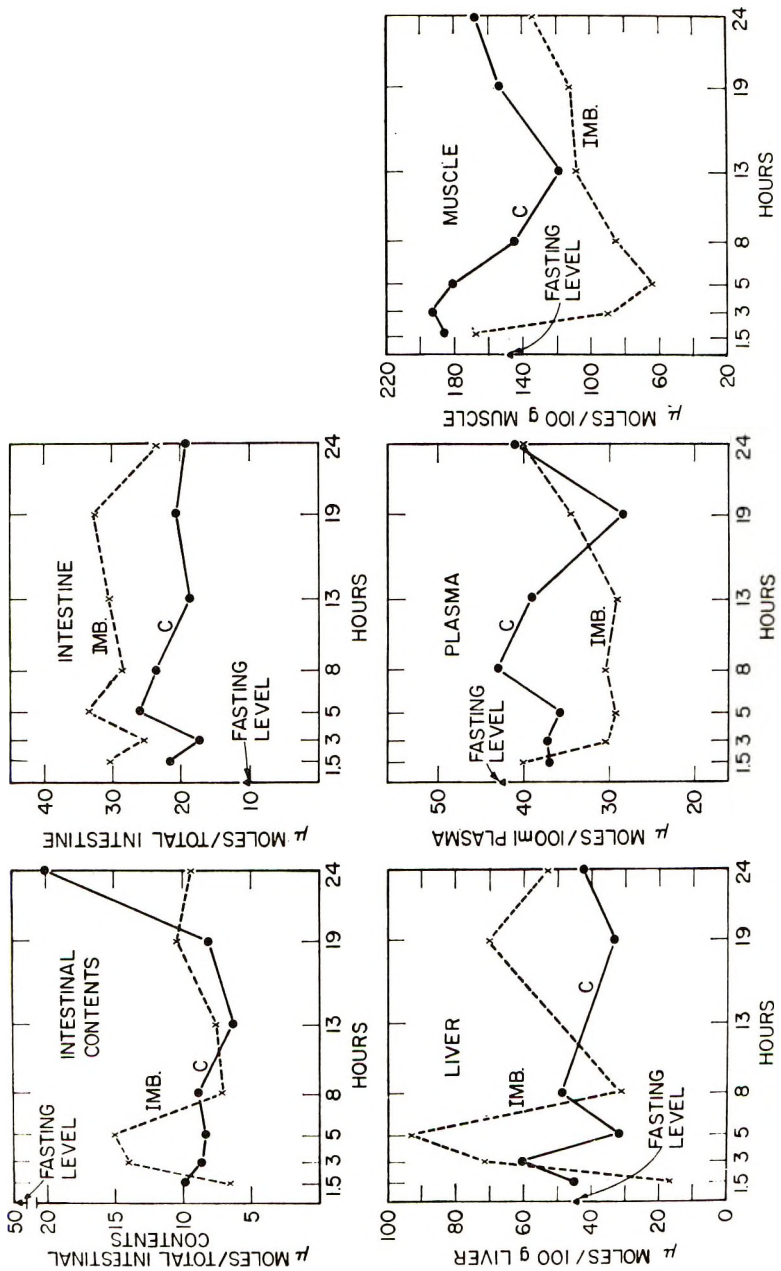

Fig. 8 Effect of amino acid imbalance on threonine concentrations in plasma and tissues of rats fed the control diet (C) and the imbalanced diet (Imb). Control diet: 6% casein plus 0.3% DL-methionine; and imbalanced diet: 6% casein plus 0.3% DL-methionine and 5.4% amino acid mixture lacking threonine.

control and imbalanced groups were similar. By 13 hours after feeding, only small amounts (1 to 3%) of the diet fed, remained in the stomach. Neither was the effect due to inhibition of absorption of threonine by the amino acids added to create the imbalance, as evidenced by the fact that the threonine concentration in the intestinal tissue of rats fed the imbalanced diet was not lower than those of the controls at any time throughout the 24-hour period. Yoshida et al. (8) have shown that the rate of absorption of threonine-U-^{14}C and histidine-U-^{14}C from the intestine of rats was not lower for the group fed threonine- or histidine-imbalanced diets than for controls. The absorptive capacity of the intestine is indeed large, and it is doubtful that absorption ever becomes limiting in a normal animal (9).

The threonine concentration in muscle of rats fed the imbalanced diet was greatly depressed 3 and 5 hours after feeding (fig. 8). The greater relative drop of threonine concentration in muscle, as compared with plasma of rats fed the imbalanced diet, may indicate that the changes in pattern of free amino acids in the plasma are a reflection of the changes in the muscle (10). Histidine, which did not increase in the plasma, even though it was added to the imbalanced diet, was lower in the muscle. Histidine is probably the second most limiting amino acid in the diet.

The results in plasma and tissue free amino acids of rats fed the threonine-imbalanced diet were in accord with those reported by Ellison and King (11), in that the most limiting amino acid, histidine, in the plasma and muscle of rats, fell below that of the controls 3 to 5 hours after the ingestion of a histidine-imbalanced diet.

The concentration of every amino acid in the liver of rats fed the imbalanced diet was higher than that of the controls 3 and 5 hours after feeding. This may be due to the liver having access to the amino acids immediately after the intestine and to the utilization of nitrogen from the excess of indispensable amino acids for the synthesis of dispensable amino acids during catabolism.

The concentration of threonine at 3 and 5 hours was substantially higher in the liver of rats fed the imbalanced diet, although the threonine intake was the same for both the control and the imbalanced groups. If the uptake of amino acids by the liver and the intestine is higher than normal, then less amino acids would be available to the plasma and the muscle. Thus, the rapid fall in threonine concentrations in plasma and muscle shortly after ingestion of the imbalanced diet may be accounted for, at least in part, by the rapid uptake of the amino acid into the liver, since the catabolism of the most limiting amino acid in animals fed the imbalanced diet was not increased (8). The increased concentrations of all the dispensable and indispensable amino acids in the liver of rats ingesting the imbalanced diet above the values for the controls would make extra substrates available for protein synthesis and may result in an increase in protein synthesis in the liver, thus, increasing the utilization of the most limiting amino acid. Sanahuja et al. (12) have reported higher protein content of the livers of rats fed an imbalanced diet. Also, isotopic studies with threonine-U-^{14}C and histidine-U-^{14}C in rats fed a threonine-imbalanced and a histidine-imbalanced diet by Yoshida et al. (8), as well as with L-histidine-U-^{14}C and L-histidine-^{15}N in rats fed different histidine-imbalanced diets by Benevenga et al. (13) and Hartman and King (14) supported this contention. Also, the incorporation of ^{14}C-labeled amino acid into liver protein was enhanced in rats force-fed a diet devoid completely of one indispensable amino acid (15).

Effects of amino acid deficiencies in depressing food intake were reported years ago by Osborne and Mendel (16) and later by Rose (17), Frazier et al. (18) and Lepkovsky (19). The depression of food intake is probably a protective response as evidenced by the observations of Sidransky and associates (20–23), that severe pathological lesions developed in rats force-fed diets completely devoid of a single indispensable amino acid, whereas rats fed similar diets ad libitum exhibited no obvious abnormalities other than those caused by a severe curtailing of their food intake. Plasma amino acid pattern of animals fed an amino acid-deficient diet is characterized by a very low concentration of the

amino acid which limits growth (24). Low levels of plasma methionine were found in chicks fed either soybean or peanut meal and the other amino acids accumulated in the circulation (25). Hill et al. (26) and Hill and Olsen (27) reported a marked decrease in the level of lysine in the plasma of chicks fed zein, and also a reduction in the level of tryptophan in chicks fed gelatin. A drop in plasma free lysine levels was also observed by Morrison et al. (28) in rats after ingestion of protein deficient in lysine.

Since the concentration of the most limiting amino acid in the plasma of rats fed the imbalanced diet fell consistently below that of the control, and the amino acid added to cause the imbalance rose during the period when food intake fell, it is thought that the addition of a surplus of all of the indispensable amino acids except one to a diet subadequate in protein might bring about some undesirable physiological or biochemical changes that would affect the feeding behavior of the animals, and that the same protective mechanism of food intake regulation might lead to reduction in food intake of rats fed an imbalanced or a deficient diet.

The concentration of threonine did not fall as markedly below that of the control values as did histidine in rats fed an imbalanced diet with histidine as the limiting amino acid (2). The less severe depression of threonine concentration may be due to the fact that the food intake of rats fed the imbalanced diet was low and the partial starvation may cause an elevation of plasma threonine toward the fasting level (27, 29, 30). As an explanation for the elevation of threonine in starved chicks, Charkey et al. (29) have pointed out that this amino acid is particularly resistant to deamination and accumulates in the blood when large amounts of amino acids are released through tissue breakdown during starvation. The slow oxidation rate of threonine, in chicks fed a complete purified diet containing free amino acids, has been demonstrated by Ousterhout (31). The fact that the tissue proteins of the rat are high in threonine (32) may also contribute to the large elevation of threonine in the blood during starvation.

The use of interval-fed animals in this experiment was aimed at equalizing the food intakes of animals fed the control or the imbalanced diet. Although interval-fed animals did not show marked food intake depression when fed a less severe threonine-imbalanced diet, the rats, nevertheless, curtailed their food intake considerably when the concentration of the imbalanced amino acid mixture lacking threonine was raised (33). Also, the food intakes of interval-fed rats, fed a histidine-imbalanced diet containing 6% casein supplemented with 0.2% L-threonine and 0.3% L-methionine and a 6% amino acid mixture lacking histidine, was depressed.[6] Thus, the phenomenon of amino acid imbalance or food intake depression occurred in interval-fed animals ingesting amino acid-imbalanced diets, and the same sequence of events observed in the plasma and tissue amino acids of rats fed amino acid-imbalanced diets ad libitum persists in animals trained to eat a single daily meal.

The concentrations of the amino acids provided in excess by the imbalanced mixture, except for that of histidine, were high in plasma at days 3 and 7. Even though food intake had increased substantially by day 22, however, the concentrations of these amino acids were not much higher at this time; some were even slightly lower, indicating that the animals were able to remove the excess amino acids from the plasma pool, possibly through increased oxidation to prevent further accumulation. The report by Klain and Winders (34) that catabolism of the imbalancing amino acids increases through enhanced oxidation in rats fed an imbalanced diet for 21 days may lend support to this idea.

The fact that histidine did not increase to the same extent in the plasma as other indispensable amino acids added to cause the imbalance in the ad libitum study may indicate that histidine is the second most limiting amino acid in the imbalanced diet. This is in accord with the findings in the experiment with rats trained to eat a single meal daily.

[6] Peng, Y., N. J. Benevenga and A. E. Harper, unpublished results.

The plasma amino acid pattern of rats pair-fed the protein-free diet against the imbalanced group appeared to be quite normal as compared with that of the control group. The most limiting amino acid, threonine, was somewhat elevated. This may be a hint as to the reason for rats selecting the protein-free diet over the imbalanced diet in the free-choice study reported previously (33). That is, the protein-free diet, though nutritionally inferior to the imbalanced diet, will result in a plasma amino acid pattern more nearly resembling that of rats fed the control diet, whereas imbalanced diets alter the plasma amino acid pattern drastically. The failure of rats to select between two balanced diets differing in nutritive value (35), would then be explained by the fact that both diets result in balanced plasma amino acid patterns. This suggests that food selection may also be regulated by some mechanism that is sensitive to the dietary amino acid pattern.

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Effect of Abomasally Infused Nitrogen Sources on Nitrogen Retention of Growing Lambs

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ABSTRACT A study involving a series of abomasal infusions was conducted to evaluate the amino acid nutritional status of lambs fed a purified diet with urea as the sole nitrogen source. When the diet was fed ad libitum the abomasal administration of casein resulted in an increase in feed consumption and an increase in nitrogen retention. When feed intake was controlled, abomasal infusion of casein increased nitrogen retention. Abomasal infusion of acid-hydrolyzed casein or a mixture of essential L-amino acids also increased nitrogen retention, but to a lesser extent than when casein was infused. When a mixture of arginine, histidine, lysine, phenylalanine and methionine was infused nitrogen retention increased, but the response was less than that observed from infusion of all 10 essential amino acids. Infusion of methionine or phenylalanine did not result in a statistically significant nitrogen retention response. In contrast, infusion of either lysine or glutamic acid increased nitrogen retention by the same magnitude as that of the mixture of the five amino acids.

Amino acid nutritional research in the ruminant has been exiguous primarily due to the unique anatomical and physiological features of the ruminant digestive tract. Many studies have indicated that the addition of supplemental amino acids to ruminant diets exert no influence on animal performance (1–4). The failure of orally administered amino acids to improve animal performance, or to detectably increase plasma amino acid concentrations (4), has been attributed to the high amino acid catabolic abilities possessed by the rumen microbes (5–8). To determine whether or not amino acids are limiting ruminant tissue growth, the amino acids must be administered in a way that renders them available to the tissues for metabolism.

Several studies involving postruminal administration of proteins have been reported. Cuthbertson and Chalmers (9) and Chalmers et al. (10) reported that casein administered duodenally was better utilized by sheep than when it was administered ruminally. Little and Mitchell (11) found that the abomasal administration of casein or soybean protein produced a greater nitrogen retention in lambs than when these same proteins were fed orally. Reis and Schinckel (12, 13) reported that the abomasal infusion of cysteine, methionine or casein resulted in a considerable increase in wool growth of mature sheep.

Feeding a purified diet to ruminants, with urea as the sole nitrogen source, provides the host animal with microbial protein as the only source of essential amino acids. Therefore, ruminants fed such a purified diet can be used to evaluate the quality of the microbial protein produced under the imposed conditions. Although Loosli et al. (14) demonstrated that all of the essential amino acids were synthesized in the rumen, Schelling et al. (4) reported that rumen microbial protein contained a lower concentration of several of the essential amino acids than a high quality protein (whole egg). Bergan et al. (15) provided evidence from studies in vitro that the amino acid composition of microbial protein and the amino acids available for absorption could be distinctly different as a result of incomplete digestion.

The objective of this study was to determine whether the abomasal administration of amino acids to lambs fed a purified diet would influence nitrogen retention.

MATERIALS AND METHODS

A series of 20 treatments of abomasal infusions of casein and amino acids using wether lambs surgically prepared with abomasal cannulas was conducted. Lambs were allowed 40 days or more to recover

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from surgery and adapt to the purified diet. The treatments were administered to animals weighing 30 to 42 kg which were kept in stanchion-type metabolism cages in a temperature-controlled laboratory.

The experimental design was a control-treatment-control cross-over design that allowed each lamb to serve as its own control. The 7-day control periods and 7-day treatment periods were separated by a 2-day adjustment period and differed only in the composition of the abomasal infusate.

During the infusion periods the abomasal cannula of each lamb was constantly connected to an infusion pump by polyethylene tubing suspended above the cage. The infusion solutions (700 ml/day) were infused continuously over a 13-hour period each day from the first feeding at 9:00 AM until the fourth feeding at 10:00 PM. The second and third daily feedings were offered at 1:00 PM and 5:30 PM. The infusion and feeding conditions were the same for all treatment and control periods except for the first two treatments and their associated controls. The lambs on the first two trials were fed only twice a day. For these trials the infusate was administered for 4 hours after each feeding.

The lambs on the first two trials were fed the purified diet on an ad libitum basis. In all other trials the lambs were control fed at a level of 6.3% of metabolic body size ($0.063\ BW^{0.75}$).

The composition of the purified diet is given in table 1, and the composition of the infusates is given in table 2. The control infusates contained only urea in an amount appropriate to make the control and treatment infusions isonitrogenous. The infusates provided nitrogen at a level equal to 0.80% (5.0% protein equivalent) of the dietary intake except for trials 1 and 2 in which the infusates supplied, respectively, protein equivalents of 2.0 and 4.0% of an estimated feed intake. The amino acids infused were all of the L-configuration. All of the infusates provided nitrogen sources in addition to, and not in place of, the nitrogen in the complete purified diet.

The criterion for evaluating the responses to treatments was nitrogen retention. The handling of samples for nitrogen-retention determinations was routine except that the feces were not heat dried.

TABLE 1

Composition of purified diet used in abomasal infusion experiments

	%
Cornstarch [1]	58.000
Cellulose [2]	30.000
Mineral mix [3]	4.500
Urea [4]	4.300
Corn oil	3.000
Choline chloride mix [5]	0.200
Ethoxyquin [6]	0.011
Vitamin mix [7]	+

[1] Powdered cornstarch, A. E. Staley Manufacturing Company, Decatur, Illinois.
[2] Solka Floc BW-40, Brown Company, Berlin, New Hampshire.
[3] Composed of: (in percent) $CaHPO_4 \cdot 2H_2O$, 34.9; KSO_4, 29.7; NaCl, 16.7; Na_2SO_4, 11.6; MgO, 4.53; $FeSO_4 \cdot 7H_2O$, 1.99; ZnO, 0.2067; MnO, 0.2000; $CuSO_4 \cdot 5H_2O$, 0.1567; KI, 0.0367; $CoSO_4 \cdot 7H_2O$, 0.0033; and Na_2SeO_4, 0.0090.
[4] Crystalline, 46% N.
[5] Choline chloride, 25%.
[6] Santoquin, Monsanto Company, St. Louis.
[7] Vitamin A palmitate, irradiated yeast and α-tocopheryl acetate to furnish 3,000 IU/kg, 385 IU/kg and 150 IU/kg of diet, respectively.

Feces were collected and frozen each day, and, upon completion of the collection period, they were blended into a homogeneous paste in a paddle-type mixer. The Kjeldahl nitrogen analyses were performed on the wet fecal samples.

RESULTS AND DISCUSSION

Since all nitrogen-retention treatment responses resulted from changes in urinary nitrogen excretion, only the treatment responses and not the complete data are presented in table 3. The treatment response was calculated by subtracting the mean nitrogen retention of the preceding and subsequent control periods from the nitrogen retention of the treatment period.

The data were analyzed by an analysis of variance which removed any replicate effect and tested the preceding control period against the subsequent control period, as well as testing the treatment period against the mean of the two control periods.

Treatments 1 and 2. These treatments involved the abomasal infusion of casein (supplemented with 4% methionine) at 2 and 4% of the estimated ad libitum feed intake. Supplementing casein with its first limiting amino acid (for some species) at the 4% level is based on previous work with casein (16–18). The nitrogen-retention responses resulting from these treatments and the other 18 treatments of this series of experiments are presented in table

TABLE 2

Composition of infusates used in abomasal infusion experiments

	Treatment identity											
	1, 2, 3, 4	5	6	7	8	9	10	14	15	16	17	18
	g [1]	g	g	g	g	g	g	g	g	g	g	g
Arginine·HCl [2]		81.6	81.6	—	—	—	81.6	—	—	—	—	—
Histidine·HCl·H_2O		64.0	64.0	—	—	—	64.0	—	—	—	—	—
Lysine·HCl		163.2	163.2	—	—	—	163.2	163.2	—	—	—	—
Tryptophan		24.0	—	—	24.0	—	—	—	—	—	24.0	—
Phenylalanine		185.6	185.6	—	—	—	185.6	—	185.6	—	—	—
Methionine	64.0	124.8	124.8	124.8	64.0	—	124.8	—	—	—	—	—
Threonine		72.0	—	—	—	—	—	—	—	—	72.0	—
Leucine		161.6	—	—	—	—	—	—	—	—	161.6	—
Isoleucine		105.6	—	—	—	—	—	—	—	—	105.6	—
Valine		118.4	—	—	—	—	—	—	—	—	118.4	—
Monosodium glutamate		—	—	—	—	432.8	432.8	—	—	—	—	—
Urea		209.6	324.9	485.0	—	423.5	248.7	457.2	477.0	423.5	393.4	510.0
NH_4Cl	—	—	—	—	—	—	—	—	—	137.1	—	—
Casein	1600.0	—	—	—	—	—	—	—	—	—	—	—
Casein hydrolysate [3]	—	—	—	—	1805.0	—	—	—	—	—	—	—
α-Ketoglutarate	—	—	—	—	—	—	—	—	—	—	—	333.2
% Nitrogen	14.50	17.19	25.34	36.65	12.73	27.36	17.65	38.7	36.2	36.62	24.78	28.43

[1] Components in each column supply the same amount of total nitrogen.
[2] All amino acids were of the L-configuration.
[3] Acid hydrolyzed.

TABLE 3

Effect of abomasal infusions on nitrogen retention

Treatment no.	No. of lambs	Treatment abomasal infusion	Nitrogen-retention responses to treatment
			g/day
1	2	Casein [1] (2% level, ad libitum feed intake)	3.67 * [2] ± 1.40 [3]
2	2	Casein [1] (4% level, ad libitum feed intake)	4.48 * ± 0.05
3	3	Casein [1] (first period)	4.56 * ± 0.60
4	3	Casein [1] (second period)	3.68 * ± 0.85
5	2	Mix 1; all essential amino acids	2.69 * ± 0.10
6	4	Mix 2; Arg, His, Lys, Phe, Met	1.12 * ± 0.58
7	3	Mix 3; Met	0.31 ± 0.30
8	2	Mix 4; casein hydrolysate [4]	2.13 * ± 0.90
9	2	Mix 6; Glu	1.46 * ± 0.63
10	2	Mix 5; Arg, His, Lys, Phe, Met, Glu	0.95 ± 0.16
11	2	Water control	−0.04 ± 0.20
12	2	Glucose (high level)	−1.50 * [5] ± 0.01
13	2	Glucose (low level)	−0.35 ± 0.66
14	2	Mix 7; Lys	1.26 * ± 0.28
15	2	Mix 8; Phe	0.55 ± 0.10
16	2	Mix 9; NH_4Cl	−0.09 ± 0.11
17	3	Mix 10; Try, Thr, Leu, Ile, Val	−0.95 ± 0.69
18	3	Mix 11; α-ketoglutarate	−0.07 ± 0.86
19	1	Mix 4; casein hydrolysate [6]	2.18
20	1	Casein; (postexperimental control)	3.42

[1] Four percent methionine added.
[2] Asterisk indicates statistical significance ($P < 0.10$); value represents the response over the mean of the two control periods. The mean nitrogen retention for all control periods was 1.19 g/lamb/day.
[3] SD.
[4] Acid hydrolyzed; methionine and tryptophan added.
[5] Lambs failed to consume feed allotment.
[6] Same mix as treatment 8 but infused at a higher level.

3. The greater nitrogen-retention value for the 4% casein infusion than for the 2% casein level is not necessarily due to the higher casein infusion. The two lambs which were administered the 4% treatment consumed more diet than the lambs infused at the 2% level; therefore, the comparison is confounded by voluntary diet intake. Voluntary feed intake increased by about 15% when the casein infusions replaced the preceding control infusion. Thus, ad libitum feed intake further confounded the experiment. In the subsequent control period, feed intake and nitrogen retention did not drop completely back to the initial level, and some lambs did not decrease feed consumption. It appeared that there was a residual effect of previous treatments influencing voluntary diet intake. The influence of infused casein on voluntary diet intake is in close agreement with the work of Egan (19), who found that duodenal casein administration increased voluntary intake of diets of low quality, low nitrogen roughage, by values ranging from 11 to 42%. He stressed the apparent importance of a chemical regulating system involving voluntary feed intake. Since the diet used in these experiments contained considerable nitrogen, it would seem that such a chemical regulating system must key on a specific nitrogen component(s), rather than on general nitrogen levels.

Treatments 3 and 4. In view of the confounding nature of the ad libitum feeding conditions experienced in treatments 1 and 2, the feeding conditions were changed to a controlled feed level based on metabolic body size. The level of feed offered was limited to ensure complete consumption. Since this amount of feed was consumed rapidly by the animals, the daily allotment was offered at four feeding periods in an effort to allow better utilization of the dietary urea nitrogen. These feeding conditions were maintained throughout the remaining treatments.

The purposes of treatments 3 and 4 were 1) to determine if a 7-day nitrogen balance period was long enough to adequately evaluate the nitrogen sources, and 2) to

determine if the carry-over nitrogen-retention effect on the subsequent control period observed in treatments 1 and 2 was due entirely to the higher feed consumption. To investigate these problems, a preceding control was followed by two consecutive, identical casein-infusion periods, and they were followed by two consecutive control periods.

The definite treatment responses for treatments 3 and 4 are presented in table 3. Although there appeared to be a slight difference between the two treatment periods, the results demonstrate that the nitrogen source was readily used in the initial 7-day nitrogen balance period. Data for both the initial and the two consecutive control periods (table 4) show that both subsequent control periods completely returned to the nitrogen-retention level of the control period preceding the treatment periods. This experiment supports the validity of using the 7-day control-treatment-control cross-over design for the evaluation of abomasally infused nitrogen sources.

Treatment 5. A mixture of all the essential [1] amino acids in the amounts to simulate casein (supplemented with 4% methionine) was infused, except that methionine in the mix provided the equivalent amount of both the methionine and cystine of casein, and phenylalanine provided the equivalent amount of both the phenylalanine and tyrosine of casein. This is justified by the known metabolic relationships of these amino acids. The amount of each amino acid infused was equivalent to that supplied by the casein infusion.

The treatment nitrogen-retention response of 2.69 g/day from the essential amino acid mix was less than the casein response. This difference in responses suggested that perhaps the nonessential amino acids were involved in the difference; or that perhaps rate of amino acid absorption was a factor, since the time for protein digestion was eliminated when the amino acid mix was infused.

Treatment 6. A mixture of arginine, histidine, lysine, phenylalanine and methionine was infused. These five amino acids were selected on the basis of the plasma amino acid values reported by Schelling et al. (4) which indicated that these five amino acids failed to increase in plasma concentration as dietary protein was increased.

The nitrogen-retention response of 1.12 g/day was less than the response to the mixture of all the essential amino acids, but was statistically significant ($P < 0.10$).

Treatment 7. Methionine was the only amino acid infused in this treatment. This experiment was the first of several designed to factor out the amino acid(s) responsible for the nitrogen-retention response observed in treatment 6. Abomasally infused methionine failed to produce a significant increase in nitrogen retention. This indicates that methionine was not the first limiting amino acid under the experimental conditions imposed.

Treatment 8. Casein hydrolysate was infused. Tryptophan was assumed to be totally destroyed by the acid hydrolysis and was therefore added to the mix along with methionine to simulate the amino acid composition of casein supplemented with 4% methionine. The amount of casein hydrolysate administered was based on the total nitrogen content, although the manufacturer of the casein hydrolysate reported that the total amino nitrogen of the hydro-

[1] Those demonstrated as being dietary essentials for maximal growth in the rat.

TABLE 4

Reproducibility of control periods

Lamb no.	Preceding control	Treatment	Treatment	First subsequent control	Second subsequent control
	Nitrogen retention, *g/day*				
4	0.50	—	—	0.89	0.62
5	0.37	—	—	0.52	0.46
7	0.66	—	—	−0.16	0.52

lysate accounted for only 76% of the total nitrogen. Therefore, the actual amounts of the amino acids infused in this treatment were somewhat lower than in the casein treatment.

The nitrogen-retention results show a response to casein hydrolysate of about the same magnitude as observed in response to the mixture of all the essential amino acids. The lower response to casein hydrolysate than to the infusion of casein suggests that possibly the rate of amino acid absorption is involved. Although the 13-hour constant infusion would tend to equalize the absorption rate of the amino acids hydrolyzed from casein and free amino acids, it is possible that the amino acid absorption from casein is distributed over the noninfusion period for a considerably longer time than it is from the casein hydrolysate. If this were the case, more efficient nitrogen utilization would possibly occur. Nonruminant work reported in a review by Mellinkoff (20) indicated that postprandial plasma amino acid concentrations were greater when free amino acids were consumed than when an equivalent amount of amino acids from protein was consumed. This suggests, as might be expected, a greater rate of absorption of the free amino acids.

Treatment 9. Glutamic acid was infused to test a source of nonessential nitrogen other than urea. The treatment nitrogen-retention response to glutamic acid was 1.46 g/day (table 3). A response to glutamic acid would not be expected, based on the supposition that the ruminant would not be lacking nonessential nitrogen in view of ruminal ammonia absorption, especially with urea as the sole dietary nitrogen source. Several possibilities seem plausible for explaining the response to the glutamic acid treatment, but subsequent treatments in this series were found to discredit two of them: 1) Glutamic acid might be serving as a nonessential amino nitrogen source, 2) it might be serving as an energy source, 3) it might influence gut absorption, 4) its carbon skeleton might influence primary routes of carbon metabolism, or 5) the amount of sodium added as monosodium glutamate might account for the response.

Treatment 10. Treatment 10 involved the same five amino acids as treatment 6, but glutamic acid was supplied in addition. The purpose was to determine if the nitrogen-retention response to glutamic acid was additive to the response observed when the five essential amino acids were infused (treatment 6).

The response to glutamic acid plus the five essential amino acids was of the same general magnitude as that observed from treatment 9 or treatment 6. Therefore, the glutamic acid response did not appear to be additive.

Treatment 11. Treatment 11 was a water infusion. The control periods contained urea as usual, and the treatment period, merely water. There was no treatment effect on nitrogen retention, indicating that urea infusion had no effect on growth.

Treatment 12. Glucose was infused to determine if the nitrogen-retention response to casein was the result of increased caloric intake. Based on similar physiological fuel values, glucose was substituted for an equal weight of casein. The treatment was made isonitrogenous to the controls by addition of urea.

Glucose infusion decreased nitrogen retention. This was the only treatment (of those experiments in which feed intake was controlled at a level less than ad libitum) in which the allotted feed was not completely consumed. Apparently glucose affects voluntary feed intake in the ruminant as in the nonruminant. The decreased nitrogen retention presumably reflects the decreased feed intake.

Treatment 13. A glucose solution lower in concentration than that used in treatment 12 was infused to indicate whether or not the increased nitrogen retention resulting from glutamic acid infusion was due to increased caloric intake. Glucose replaced glutamic acid on an equal weight basis. The treatment was made isonitrogenous to the controls by the addition of urea.

The low level of glucose infusion had no statistically significant effect on nitrogen retention (table 3). The results of this treatment discredit the possibility that the glutamic acid response was due to an increased caloric intake.

Treatment 14. Lysine was infused to continue factoring out the amino acid(s) responsible for the nitrogen-retention increase resulting from treatment 6.

Infused lysine gave a nitrogen-retention response of about the same magnitude as treatment 6. Based on the results to this point, lysine is implicated as the first limiting amino acid for the ruminant under the experimental conditions imposed.

Treatment 15. In a continued effort to factor out the amino acid(s) influencing the response obtained in treatment 6, phenylalanine was infused. The nitrogen-retention response value of 0.55 g/day was not statistically significant.

Treatment 16. Ammonium chloride was infused to furnish the same amount of nitrogen as supplied by glutamic acid in treatment 9. This was infused to determine if a quantity of ammonium ion isonitrogenous to glutamic acid would produce a nitrogen-retention response. As indicated in table 3, ammonium chloride produced no nitrogen-retention response.

Treatment 17. A mixture of tryptophan, threonine, leucine, isoleucine and valine was infused. These represent the remaining essential amino acids not infused in treatment 6.

Because of considerable variability in the control periods, the negative response of − 0.95 g N/day was not statistically significant. Clearly, there was no positive effect on nitrogen retention. This experiment indicates that these five amino acids do not include the first limiting amino acid.

Treatment 18. α-Ketoglutarate was infused to ascertain whether the carbon skeleton of glutamic acid would produce any response. No response was obtained.

Treatment 19. As discussed for treatment 8, the amount of amino nitrogen in the acid-hydrolyzed casein was less than the total nitrogen content. In this experiment the amount of casein hydrolysate infused was increased so that the amount of amino nitrogen administered was equivalent (based on data supplied by the manufacturer) to that administered by the casein infusion.

Unfortunately, nitrogen retention of only one lamb was available in this treatment. The response of 2.18 g/day was very similar to that obtained at the lower level of infusion (treatment 8). This suggests that the lower amino nitrogen level in the previous casein hydrolysate infusion was probably not the factor responsible for the response being lower than that of casein.

Treatment 20. Casein was infused into one of the lambs which had been subjected to the most treatments to verify its competency to respond to nitrogen infusion. The nitrogen-retention response of 3.42 compares favorably with the initial casein infusions and demonstrates the capability of the lamb to respond to treatments.

In evaluating the microbial protein with experiments such as these, the source(s) of microbial protein is undoubtedly an important consideration. The absence of a significant protozoan population in the rumen of lambs fed the purified diet was verified by microscopic examination of rumen contents; therefore, the protein evaluated was bacterial in origin. Schelling et al.[2] found that a limited number of bacterial species were present in the rumen of mature sheep when purified diets were fed. The organisms present, however, were species that are normally present in the rumen. The absence of a thriving rumen protozoan population also is often encountered when high grain diets are fed. Because of the differences in ruminal conditions between the purified diet and other diets, the authors would caution against direct extrapolation of the results of this study to animals exposed to different dietary conditions.

Although the trials reported here have only begun this type of study of ruminant amino acid nutrition, the treatments have demonstrated that amino acid nutrition is a factor limiting the growth of lambs fed a purified diet with urea as the sole nitrogen source. Not only was animal growth, as measured by nitrogen retention, influenced by amino acid administration, but also voluntary feed intake was influenced by the administration of amino acids in protein form.

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RNA Polymerase Activities and Other Aspects of Hepatic Protein Synthesis During Early Protein Depletion in the Rat [1,2]

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ABSTRACT Certain aspects of the hepatic protein-synthesizing system were studied in vivo or in vitro in young male rats fed diets with various levels (0 to 40%) of casein for periods up to 42 days. First, the incorporation of L-leucine-1-^{14}C or L-methionine-^{35}S into microsomal protein was established to be related inversely to the protein level of the diet; data in vitro indicated these differences were more closely related to the capacity of the microsomal fraction. A similar inverse relationship was shown for RNA synthesis in vivo; the specific activity of ^{14}C-incorporation (using orotic acid) into the total RNA of various subcellular fractions was found to be greatest in the nuclear isolate. To determine the nature of this phenomenon, the activity of DNA-dependent RNA polymerase was examined; two proposed polymerase assay methods were used, a conventional Mg^{2+}-dependent or a Mn^{2+}-$(NH_4)_2SO_4$ system. The activity of the former system was found to be significantly higher in nuclei from rats fed the low protein diets and lower in the high protein group, whereas the latter system was unresponsive to the dietary protein level. Finally, liver polysomal profiles from rats fed a low protein for 14 days showed a relatively larger proportion of polysomal aggregates with a decrease in monosomes. Over a period of prolonged depletion, however, the relatively higher levels of protein and RNA synthesis in the low protein groups diminished, indicating an inevitable breakdown in this adaptative phenomenon.

In attempts to understand the regulation of the mammalian protein-synthesizing system, various morphological and chemical features of protein and RNA metabolism have been compared in hepatic tissue under a variety of experimental conditions. For example, protein depletion has been extensively employed for this purpose and remains a very useful approach. Herdson et al. (1) and Svoboda and Higginson (2) described some of the ultrastructural changes in the rat liver following prolonged protein deficiencies while other investigators reported various chemical and enzymatic changes under similar dietary conditions (3–6). Of more pertinence to the present study, there is evidence of a higher specific activity of incorporation of labeled amino acids into liver proteins in vivo (7–10), or of ^{32}P into certain RNA fractions (11, 12), from rats fed inadequate amounts of protein.

In the present report, the effects of the level of dietary protein on some of the earliest biochemical events associated with protein metabolism in hepatic tissue were studied further. Since DNA-dependent RNA polymerase is a primary index of protein and RNA metabolism, this enzyme activity was measured in nuclei from animals fed inadequate to excessive amounts of protein for various periods of time. To evaluate the overall significance of such RNA polymerase changes, other related aspects of the protein synthesizing apparatus were also determined in vitro and in vivo under similar conditions. Certain of these biochemical changes were compared sequentially up to 42 days of protein depletion to study the control mechanism and the limits within which such systems

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[2] Part of these data were included in a preliminary report at the 1967 Annual Meeting of the Federation of American Societies for Experimental Biology in Chicago, Illinois.

can function before irreversible pathological changes occur. Two RNA polymerase activities were studied, a conventional Mg^{2+}-dependent system as well as a Mn^{2+}-dependent $(NH_4)_2SO_4$-stimulated system (13, 14). These and other approaches, including polysomal profile analyses, are included.

MATERIALS AND METHODS

Young (42 days of age) male albino rats of the Sprague-Dawley strain had been individually caged in a temperature- and humidity-controlled room and fed commercial laboratory ration [3] prior to being placed on experimental diets containing either zero, 5, 10, 20 or 40% protein (casein). The casein content of the diet was varied at the expense of the cornstarch (7.8 to 47.8%) (table 1). During the experimental periods, the animals were always fed ad libitum and then fasted overnight (18 to 20 hours) before being killed; tissue samples were always maintained at 0 to 4°, unless otherwise specified. A total of 248 rats was required for these studies.

Amino acid incorporation into microsomal protein in vivo. To determine amino acid incorporation into microsomal protein in vivo, 103 rats were fed 5, 10, 20 or 40% casein diets.

In the first experimental trial, animals were fed these diets for 28 days and then injected with L-methionine-^{35}S (7 μCi/100 g body weight) [4] and killed 40 minutes later. Each liver was perfused in situ with ice-cold sterile saline. A portion of liver was minced and then homogenized (TRI-R tissue homogenizer) in ice-cold 0.25 M sucrose (2.5 ml of 0.25 M sucrose/g liver). The remaining tissue was frozen with liquid nitrogen and stored. The homogenate was centrifuged at 15,000 × *g* (0°) for 10 minutes in an International Model HR-1. A fat-free supernatant fraction was then spun at 105,000 × *g* (0°) in an International BD-2 for 1 hour to obtain the microsomal pellet. After this pellet was washed with cold 0.25 M sucrose, 10% trichloroacetic acid (TCA) was added to precipitate the total protein, which was subsequently purified by Schneider's fractionation procedure (15). Two aliquots of a 0.1 N NaOH digest of the purified protein were taken, one for a protein determination. The other was saved for the determination of radioactivity.

In another trial, changes in amino acid incorporation into microsomal protein in vivo were also studied after 3, 7, 14 or 28 days of dietary treatment (5, 10, 20 or 40% casein). After an overnight fast, each animal was then injected intravenously with L-leucine-^{14}C (5 μCi/100 g body weight) [5] and killed exactly 60 minutes later.

RNA synthesis in vivo in various subcellular fractions following 6-^{14}C-orotic acid

[3] Purina Laboratory Rat Chow, Ralston Purina Company, St. Louis.

[4] L-Methionine-^{35}S (specific activity, 25.8 μCi/μmole) and adenosine 5'triphosphate-8-^{14}C disodium salt (ATP-^{14}C) (specific activity, 18 μCi/μmole) were purchased from Schwarz BioResearch, Inc., Orangeburg, New York.

[5] L-Leucine-1-^{14}C (specific activity, 25 μCi/μmole) and orotic acid-6-^{14}C (specific activity, 4.9 μCi/μmole) were purchased from New England Nuclear Corporation, Boston, Massachusetts.

TABLE 1

Composition of diets [1]

Casein, %	0.0	5.0	10.0	20.0	40.0
Corn oil	5.0	5.0	5.0	5.0	5.0
Salt mixture [2]	4.0	4.0	4.0	4.0	4.0
Choline chloride	0.2	0.2	0.2	0.2	0.2
Inositol	0.1	0.1	0.1	0.1	0.1
Vitamin mixture [3]	0.1	0.1	0.1	0.1	0.1
Dextrin	21.4	21.4	21.4	21.4	21.4
Sucrose	21.4	21.4	21.4	21.4	21.4
Cornstarch	47.8	42.8	37.8	27.8	7.8

[1] All dietary ingredients, with the exception of the corn oil (Corn Products Company, New York) and sucrose, were purchased from Nutritional Biochemicals Corporation, Cleveland.

[2] Hegsted salt mixture 1941 J. Biol. Chem., *138*: 459.

[3] Vitamin mixture contained per kg of diet: (in milligrams) thiamine, 5.0; riboflavin, 5.0; niacin, 80.0; Ca pantothenate, 50.0; folic acid, 0.25; pyridoxine·HCl, 4.0; biotin, 0.20; menadione, 5.0; vitamin E acetate, 400.0; and vitamin A, 13,000 IU; vitamin D, 2,000 IU; mixture made up to 1 g with dextrose.

administration. Eighteen animals were fed 5 or 20% casein diets for 14 or 28 days. After an overnight fast, each rat was injected intraperitoneally with 6-^{14}C-orotic acid (5 μCi/100 g) [6] and killed exactly 40 minutes later. The isolation of the nuclear, cytoplasmic, microsomal or mitochondrial subfractions was carried out as described by Tata and Widnell (14). In addition, nuclear, nucleoplasmic and nucleolar residue fractions from another aliquot from these tissues were isolated using the method of Munro et al. (16). A comparison between these two fractionation methods (for whole nuclear specific activity) gave comparable results.

Hepatic protein synthesis in vitro. Eight animals were fed either 5 or 20% casein diets for 28 days; then, after an overnight fast, the microsomal fractions of the liver tissue were isolated as described above. The final incubation medium contained 0.4 ml of the 105,000 × *g* supernatant (cell sap), 0.4 ml of microsomal suspension and 0.2 ml of supporting medium. This 1.0 ml suspension (pH 7.6) contained 1 μM ATP, 0.25 μM GTP, 5 μM phospholenolpyruvate (PEP), 50 μg pyruvate kinase, and 0.4 μCi of L-leucine-1-^{14}C. After the samples were incubated for 20 minutes at 37°, the reaction was stopped by the addition of cold 10% TCA. The specific activity of the isolated proteins, as well as the protein concentration, was determined as described above.

DNA-dependent RNA polymerase activity. Sixty-three animals were fed for 28 days on diets containing 5, 10, 20 or 40% casein. After killing, each liver was immediately perfused with ice-cold sterile saline in situ, removed, and 3-g duplicate samples taken. Weiss' enzyme aggregate (17) was prepared with modifications as described recently (18). The activity of RNA polymerase was determined by the incorporation of 8-^{14}C-ATP[2] into RNA. The protein remaining after the removal of the labeled RNA was isolated according to Schneider's fractionation procedure (15) and 0.5 ml of 0.2 N NaOH was added to dissolve the dry protein pellet. The sample was then diluted with water to 1 ml and an aliquot (0.5 ml) was taken for protein determination.

The change in RNA polymerase activity in 24 additional animals fed zero, 5 or 20% casein diet for either 1-, 2-, 4-, or 6-week periods was compared in another experiment using the above techniques.

The RNA polymerase activity was also determined in the nuclei from another 8 animals maintained on either 5 or 20% casein diets for 28 days to compare the Mn^{2+}-dependent-$(NH_4)_2SO_4$-stimulated system, based on the methods of Widnell and Tata (13) and Tata and Widnell (14), with the Mg^{2+}-dependent system described above. The samples for the Mg^{2+}-dependent system were incubated for 5-, 10-, 16-, 21- and 30-minute periods. A 15-minute preincubation period for the Mn^{2+}-$(NH_4)_2SO_4$ system with unlabeled ATP was followed by an incubation with the labeled ATP. Measurements were made at 15-, 30- and 45-minute periods. The detailed procedures and analyses have been described (18).

Polysome profiles. Monosomes and polysomes from animals fed either the 5 or 20% casein diets for 14 days were prepared by the method of Drysdale and Munro (19). The isolated suspensions were layered on a 4.6 ml linear sucrose gradient (10 to 40%) and centrifuged at 0° for 10 minutes at 38,000 rpm in an ultracentrifuge [7] with a SW-50 rotor. After centrifugation, the gradient was collected by puncturing the bottom of the tube with a needle. The absorbancy of the collected material was determined at 260 mμ using a recording spectrophotometer [8] with a flow-through cell system.

Other analyses. All radioactive samples were plated according to a modified method described by Siekevitz (20) on stainless steel planchets, and their activity determined by a gas-flow counter.[9] Protein concentrations were determined by the method of Lowry et al. (21) using bovine albumin as a standard. The RNA concentration was determined by the method of Fleck and Begg (22). DNA determinations were based on the Giles and Myers modification (23) of the Burton procedure. Calf thymus DNA was used as the standard.

[6] See footnote 5.
[7] Spinco, Model L-2, Beckman Instruments, Inc., Fullerton, California.
[8] Gilford, Model 2000.
[9] Nuclear-Chicago Corporation, Des Plaines, Illinois.

TABLE 2

Comparison of protein synthesis in vivo with RNA polymerase activity in vitro — 28 days of dietary treatment

Level of dietary protein	^{35}S-methionine incorporation into microsomal protein in vivo [1]	DNA-dependent RNA polymerase activities (8-^{14}C-ATP incorporation into nuclear RNA in vitro) [2]
%		
5	117 ± 1.5 [3]	137 ± 6.1 [3]
10	115 ± 1.4	121 ± 4.6
20	100 ± 0.7	100 ± 1.7
40	93 ± 0.6	85 ± 0.7

[1] Actual mean value of the control group (20% protein) = 1093 cmp/mg protein. A total of 72 animals was used in this part of the experiment.
[2] Actual mean value of the control group (20% protein) = 10.3 cpm/μg DNA. A total of 63 animals was in this part of the experiment.
[3] Mean ± SE of the mean; underlined values were significantly different from the control values (20% group) $P < 0.01$.

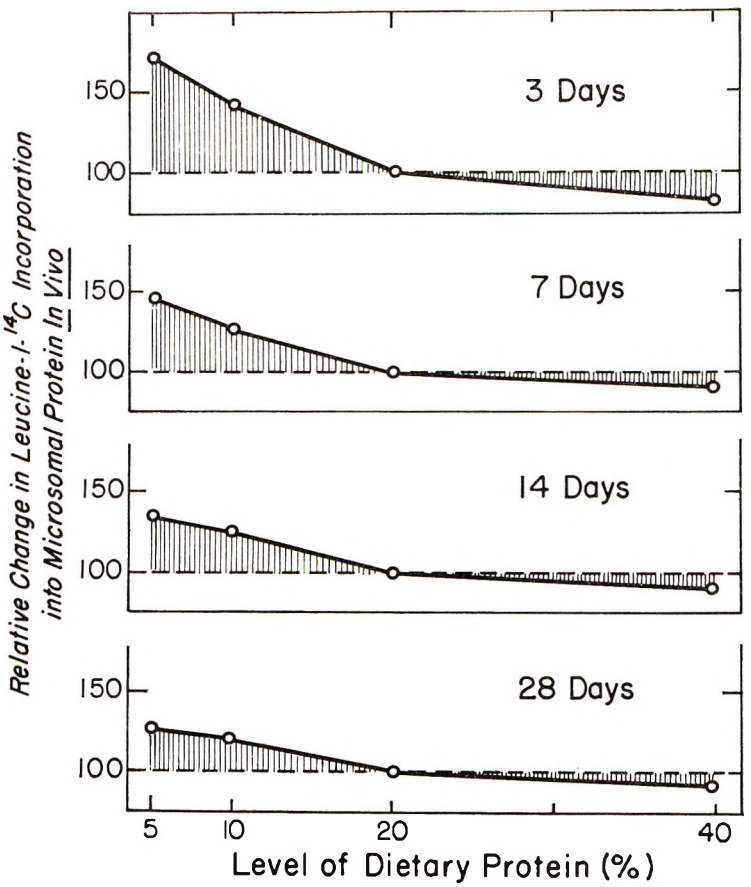

Fig. 1 Hepatic protein synthesis in vivo. The 100% line for each day of killing is based on the mean value for the control rats (20% casein). These mean values were derived from counts per minute per milligram of microsomal protein. The actual values were approximately 6500 cpm/mg protein for the control groups.

TABLE 3

Hepatic protein synthesis in vitro [1]

Mixture	Source of incubation components		Relative level of ^{14}C-leucine incorporation
	Microsomes	105,000 × *g* cell sap	
	%	%	%
1	20	20	100 [2]
2	5	5	141 [3]
3	5	20	132 [3]
4	20	5	105

[1] "Cross-over" experiment.
[2] Derived from 102 cpm/mg microsomal protein; 20% = casein in diet for 28 days; 5% = casein in diet for 28 days. Underlined values are significantly different from mixture 1. These relative values were calculated and expressed as percent for each trial and averaged for the above comparisons since each trial was carried out on a separate day. Each trial was carried out in duplicate and represented tissue from 1 rat from each dietary group. The 8 pooled results for each mixture were derived from 4 rats from each dietary group. This procedure has been described in earlier publications from this laboratory (38).
[3] Underlined values are significantly different from mixture 1 ($P < 0.01$).

In samples designated for both protein and nucleic acid determinations, the nucleic acids were first extracted; the remaining protein was then purified by Schneider's fractionation procedure (15) and digested by an appropriate amount of 0.1 N NaOH before analysis.

RESULTS

Amino acid incorporation into microsomal protein in vivo. The ability of rats to incorporate labeled amino acids into hepatic microsomal protein is related inversely to the protein level of the diet (table 2). This observation was independent of the experimental variables in this particular study: duration of the dietary period; the isotope used (L-leucine-^{14}C or L-methionine-^{35}S); and time of injection (40 or 60 minutes). It is of particular interest that the differences noted among the various experimental groups tended to diminish with time (fig. 1).

Hepatic protein synthesis in vitro. Liver fractions from protein-depleted animals also show a stimulation for the incorporation of amino acids into microsomal protein (i.e., specific activity) in vitro, in support of the above observations in vivo. This increased capacity, however, appears to be largely attributed to the microsomal fraction, whereas the cell sap fraction con-

TABLE 4

6-^{14}C-orotic "incorporation" into RNA in vivo — 14 days of dietary treatment

Level of casein	Specific activities				
	Cytoplasmic	Mitochondrial	Microsomal	Whole nuclei [2]	Nucleolar [2]
%	*cpm/mg RNA-P*				
5	221 ± 9 [1]	35 ± 5	124 ± 6	2940 ± 40	4440 ± 90
20	101 ± 16	26 ± 7	69 ± 4	1270 ± 38	1705 ± 30

[1] Mean ± SE of mean; duplicate liver samples were derived from 4 to 6 rats from each group. Underlined values are significantly different from the 20% casein values ($P < 0.01$).
[2] Nuclear and nucleolar fractions were not derived from the same animals used for the microsomal, mitochondrial and cytoplasmic fractions. Animals were killed 40 minutes after receiving labeled orotic acid.

TABLE 5

6-^{14}C-orotic "incorporation" into nuclear RNAs in vivo — 28 days of dietary treatment

Level of casein	Specific activities			
	Whole nuclei	Nucleoplasmic		Nucleolar
		NaCl extract	PO_4 extract	
%	*cpm/mg RNA-P*			
5	2261 ± 45 [1]	1851 ± 31	1348 ± 27	2785 ± 40
20	1061 ± 23	911 ± 20	734 ± 25	1432 ± 33

[1] Mean ± SE of mean; duplicate liver samples were derived from 4 rats from each group. Underlined values are significantly different from respective controls ($P < 0.01$).
[2] These nuclear fractions were obtained by the same method employed for the nuclear and nucleolar fractions in table 4. Animals were killed 40 minutes after receiving labeled orotic acid.

tributes relatively little to this phenomenon (table 3). This type of "crossover" technique obviates considerations, such as amino acid pool sizes, which may be a factor in interpreting studies in vivo.

Specific activity of the RNA from various subcellular fractions following the injection of 6-^{14}C-orotic acid. The variation in labeling the RNA in nuclear and cytoplasmic fractions is considered indicative of the general course of RNA synthesis (24). The specific activity of label in the RNA from the protein-depleted rats was significantly greater than seen in control rats, especially in the nucleus, and in particular, in the nucleolar residue. In agreement with the above data on amino acid incorporation, these differences appear to diminish if one compares the 14- to 28-day periods for the nuclear fractions (tables 4 and 5).

DNA-dependent RNA polymerase activity. The activity of RNA polymerase was also related inversely to the dietary protein content among rats fed 5, 10, 20 or 40% casein diets (table 2). Again, the differences in enzyme activity among these

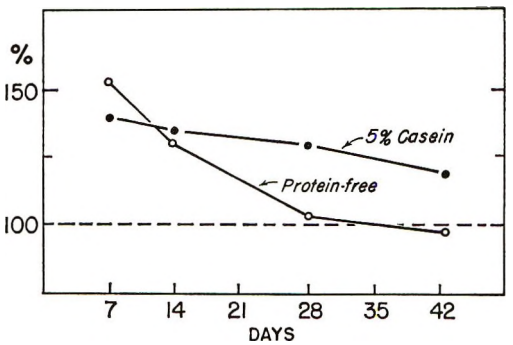

Fig. 2 Comparison of DNA-dependent RNA polymerase activity in rats fed zero, 5 or 20% casein diets for 7, 14, 28 or 42 days. The 100% line refers to enzyme activity (based on counts per minute per milligram DNA-P of ^{14}C-ATP incorporation into nuclear RNA in vitro) of rats fed "control" diet (20% casein).

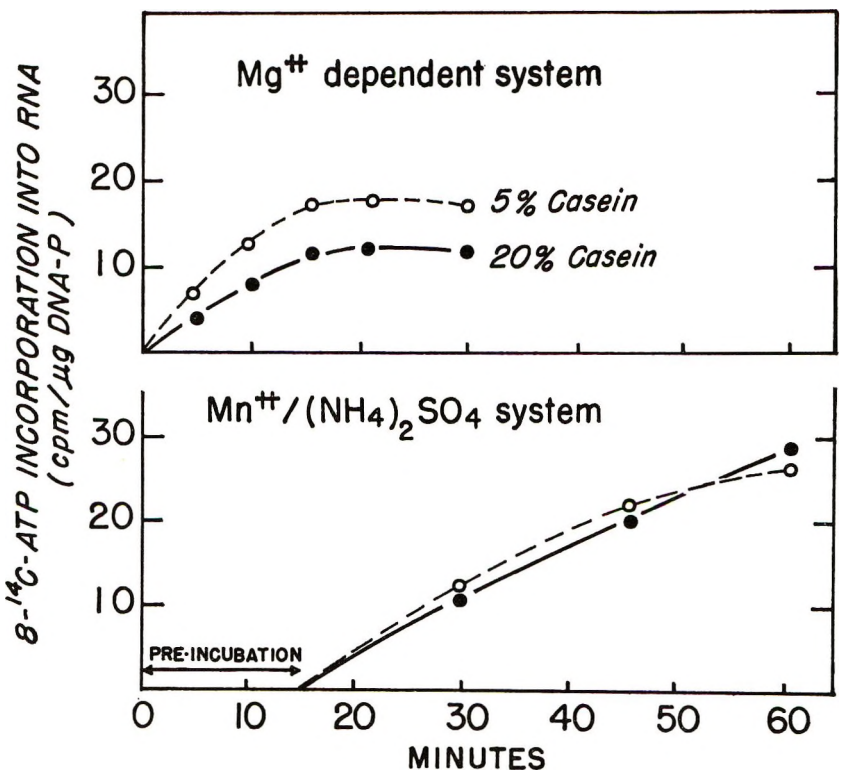

Fig. 3 Comparison of two systems for measuring DNA-dependent RNA polymerase activity. The rats were fed either 5 or 20% casein diets for 28 days. The Mn^{2+}-$(NH_4)_2SO_4$ system was preincubated for 15 minutes with unlabeled ATP. (See Materials and Methods.)

groups tended to diminish over the 42-day period in agreement with all the data in vivo. In support of this phenomenon, the values of the protein-free group diminished more rapidly than those of the 5% casein group (fig. 2). Nuclear DNA content was used as a preferential reference for enzyme activity since DNA remains relatively constant over an extended period of protein deprivation (4, 25).

In the kinetic studies comparing the Mg^{2+}-dependent and the Mn^{2+}-$(NH_4)_2SO_4$-dependent system, only the Mg^{2+}-dependent system was responsive to the effect of the diet (fig. 3).

Polysome profiles. From the polysome profile analyses of animals fed 5% casein diets for 14 days, there appears to be a relative increase in polysomes, and a decrease in monosomes and possibly oligosomes, when compared with the controls, the 20% casein group (fig. 4).

DISCUSSION

The protein-synthesizing system is adaptable to wide fluctuations in the availability of dietary protein. When the quantity of protein is inadequate, it is apparent that a series of responses and adaptations takes place in the liver to delay the well-documented pathological changes. These adaptations are evident in selected nuclear and cytoplasmic events related to protein synthesis. For example, protein-depleted rats show an increased incorporation of labeled precursors (using ^{14}C-orotic acid)

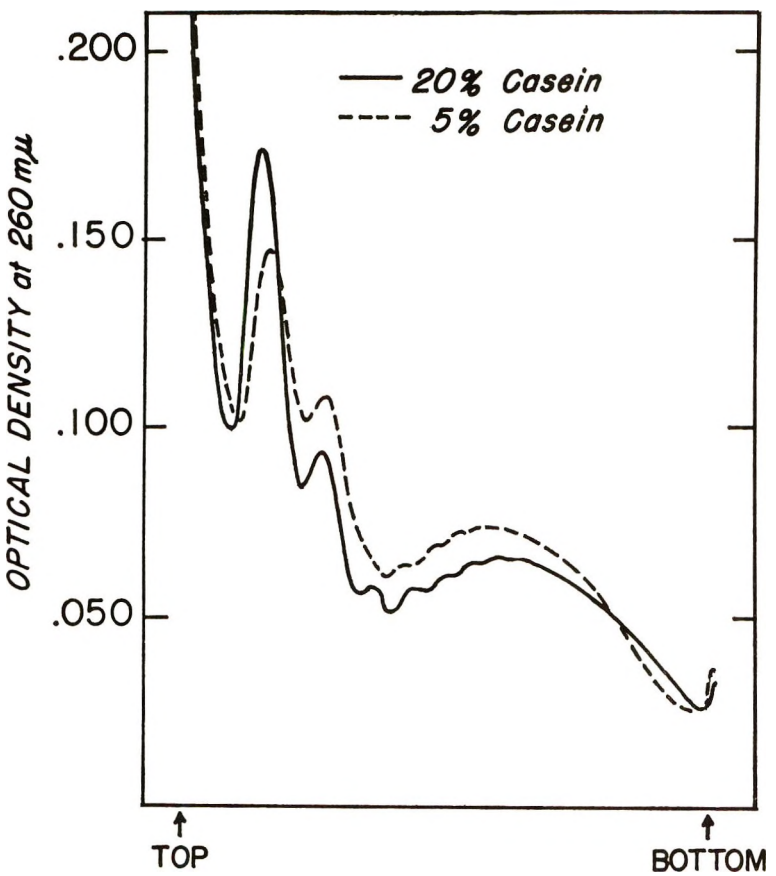

Fig. 4 Typical polysomal profiles from rats fed either 5 or 20% casein for 14 days. The monosomal peak is on the left side of the tracing, the polysomal area occupies the right half of the tracing. The samples were layered over the sucrose gradient after being equilibrated in terms of optical density.

into the total RNA isolated in various hepatic fractions (especially the nuclear and the nucleolar). Stenram (26) obtained similar results using tritiated cytidine, and noted that the enlargement of the nucleolus was due primarily to its increased RNA content. These observations are not unequivocally supported by Munro and coworkers (16), who recalculated the specific activity of adenine incorporation into RNA in terms of total nuclear RNA activity. Recently, Girija et al. (12) showed that in protein-depleted animals, labeled RNA precursors were actually taken up for the most part by a so-called rapidly labeled form of RNA. According to Georgiev (24), this rapidly labeled RNA localizes in the nucleolochromosomal complex and is represented by heavy precursors of rRNA, heavy polycistronic dRNA, and newly formed tRNA. Both the tRNA and rRNA species are known to be synthesized in the nucleolus (27) which occupies a central position in the regulation of cytoplasmic RNA and protein metabolism.

In a preliminary report, Fillios and Shaw [10] showed that the activity of Weiss' aggregate enzyme (17) was correlated inversely to the protein content of the experimental diet, suggesting a possible derepression phenomenon during early protein depletion. Others (28) support these findings. The RNA product of this Mg^{2+}-dependent enzyme system has been purported to be associated with the synthesis of a ribosomal-like RNA (29). On the other hand, a Mn^{2+}-$(NH_4)_2SO_4$-dependent system was postulated to be associated with the synthesis of a more DNA-like RNA (29), but its physiological significance has yet to be ascertained. In the present studies and in others from our laboratory (18, 30), the activity of this latter system always has been unresponsive to dietary manipulations. The questionable physiological significance of the $(NH_4)_2SO_3$-stimulated system has been discussed elsewhere (18).

The importance of extranuclear control mechanisms should also be kept in mind. Several years ago, Kosterlitz (3) characterized certain chemical changes in the cytoplasm during protein depletion. Later, more specific observations were made by Wikramanayake et al. (6) who reported an extensive loss of total microsomal RNA and protein. Despite such losses, protein-depleted animals show an increased facility to incorporate labeled amino acids into the remaining microsomal protein in vivo, as emphasized by the present data. Gaetani et al. (31) attributed the in vivo phenomena to the increased activity of the amino acid-activating enzymes of the cell sap. But our studies in vitro (table 3) suggest that the cell sap fraction from protein-depleted animals possesses only a slightly enhanced activity and certainly not of a magnitude to explain the data in vivo. Munro et al. (32) and Hird et al. (33) associated the increased incorporation of ^{14}C-leucine into a cell sap fraction with the activity of a postmicrosomal fraction. This latter fraction contains the amino acid-activating enzymes, but they found no correlation between the two activities. Of perhaps more direct relevance is the fact that the polysomal profile analyses show that rats fed a low protein diet during the early phase (14 days) do not show a reduction of, but actually have relatively more, polysomes as compared with monosomes. This appears to be contrary to the claims of Mandel et al. (34) who contended that rats fed low protein diets show a decrease in ^{14}C-leucine incorporation in vitro, as well as a relative decrease in polysomes and an increase in monosomes. (The period of protein depletion in their experiments was in excess of 5 weeks.) In studies on amino acid deficiencies, Fleck et al. (35) found an increased oligosome (including monomer) population in livers from rats fed a diet deficient in tryptophan. This observation was accompanied by a decreased specific activity of amino acids into microsomal protein in vitro. This effect has been confirmed in mice (36). The protein of the polysomes and postmicrosomal pellet of livers from such animals also showed a decreased incorporation of ^{14}C-leucine or ^{14}C-tryptophan in vitro, as compared with controls.[11] Sidransky et al. (37), however, found an enhanced amino acid incorporation into liver and plasma protein in vivo

[10] Fillios, L. C., and C. Shaw 1967 Hepatic RNA polymerase activity and dietary protein. Federation Proc., 26: 409 (abstract).

[11] Wunner, W. H., J. Bell and H. N. Munro 1966 The response of rat liver polysomes to complete and tryptophan-deficient amino acid mixtures. Federation Proc., 25: 239 (abstract).

in rats fed threonine-deficient diets and observed an increase in the larger ribosomal aggregates (polysomes) as well as a somewhat greater amino acid incorporating activity of the supernatant fraction. Furthermore, cholesterol feeding depressed amino acid incorporation into microsomal protein in vivo or in vitro (38); such diets increased the relative percentage of oligosomes at the expense of larger aggregates (39); RNA polymerase activity in vitro, as well as the incorporation of 6-^{14}C-orotic acid into the nucleolar residue in vivo, was similarly decreased (18, 30). Although the experimental conditions for all these studies are quite different, the data at least demonstrated a consistent relationship between morphological (polysomal profile) and biochemical responses (amino acid incorporation, etc.).

To appreciate the adaptive mechanisms, it is useful to follow the biochemical changes over an extended period of early protein depletion. We carried out our studies up to 6 weeks; and the observed higher specific activities among the rats fed low protein or protein-free diets tended to diminish with prolonged dietary treatment (RNA polymerase activity, precursor incorporation into the nuclear RNA in vivo and amino acid incorporation into microsomal protein in vivo). Within a matter of perhaps a few hours, the liver cell carries out a remarkable reorganization of its resources to delay pathological changes. During the first few days this adaptation is quite impressive, as shown by the protein synthetic activity, even though the total cytoplasmic RNA protein and ribosomal population is actually being reduced. This enhanced ability to incorporate labeled amino acids into protein should not be explained by changes in amino acid pool size (as indicated by the crossover experiments in vitro). Thus, the system is able to utilize its ever decreasing resources to the fullest. In the nucleolus, it is presumed that ribosomal precursors are accumulating for the rapid restoration of a normal complement of polysomes at the endoplasmic reticulum, etc., once the exogenous amino acid supply is restored to normal.

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Influence of Body Composition, Methionine Deficiency or Toxicity and Ambient Temperature on Feed Intake in the Chick [1,2]

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ABSTRACT Three studies were conducted to investigate the effect of methionine deficiency or toxicity, initial body composition and temperature on voluntary food consumption and body weight gain of chicks. Either a deficiency or an excess of methionine depressed both the amount of food consumed and body weight gain. Chicks maintained at a low temperature, regardless of their initial body composition, were able to increase both their consumption of a low methionine diet and their weight gain. Obese chicks consumed significantly less food than their nonobese counterparts, regardless of the ambient temperature or the methionine content of the diet. Similarly, the growth rate of the obese chicks was lower than that of the nonobese ones fed the same diet. Obese chicks fed an adequate methionine diet tended to consume nearly normal amounts of food, gain weight, lose their body fat and return to a normal body composition within approximately 2 weeks. The body composition appeared to exert an important role in regulating the voluntary food intake of the chick.

Obese children have been observed to eat less and spend less time in exercise than normal weight controls (1, 2). Intake per kilogram of lean body weight for both sexes of teen-agers was similar except for the extremely obese, who showed a sudden drop. It would appear that subjects who are excessively obese might not be able to rely on caloric restriction to achieve weight loss (3).

Anderson et al. (4) found that the addition of 4% methionine to the basal diet caused a marked depression in food intake and growth of chicks. Rats exposed to a cold environment increased their food consumption of an amino acid-imbalanced diet as well as their weight gain (5, 6).

Rats that were force-fed, then abruptly allowed free access to food, either did not eat or ate sparingly until their body weight declined to approximately that of the normal weight controls. Although the body weight of these formerly obese rats appeared nearly normal, carcass analysis showed that they still had an excess of body fat (7).

The experiments reported are an attempt to evaluate the effect of initial body composition on physiological appetite as measured by food consumption and body weight gain.

MATERIALS AND METHODS

One-day-old Arbor Acre male chicks were fed a regular chick starter for 8 days in experiment 1 and for 6 days in experiments 2 and 3. The chicks then were randomly divided into two subgroups. One group was fed a low protein (6.4%), high energy diet ad libitum, the other was fed restricted amounts of a high protein (44.0%) diet, shown in table 1, for 9 days in experiment 1 and for 14 days in experiments 2 and 3. In this manner chicks of similar weights and age but with markedly different body composition were produced. In experiments 2 and 3, the chicks on the high protein diet were fed ad libitum for the last 3 days of the second preliminary period, because it was felt that some of the marked differences in food intake obtained in experiment 1 may have been due to initial hunger. In experiment 1, the chicks from each of the body composition groups were

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[2] Portion of a dissertation presented by the senior author as partial fulfillment of requirements for the Ph.D. degree to the Graduate School of the University of Maryland.
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TABLE 1

Composition of diets

	Low protein, 6.4%	High protein, 44%	Methionine-deficient
	%	%	%
Fish meal	0.50	3.45	
Soybean meal (50%)	12.00	82.90	10.00
Glucose monohydrate [1]	27.40		48.24
Cellulose [2]	5.16		
Isolated soybean protein [3]			24.00
Dried fermentation solubles [4]			0.25
Starch	30.00		
Soybean oil	14.00	3.45	2.50
Corn oil			2.50
Fish oil			0.50
Hydrolyzed animal and vegetable fat [5]			4.50
Sand	5.16		
Limestone	0.90	1.55	1.00
Dicalcium phosphate dihydrate	2.40	4.15	2.75
Sodium chloride	0.40	0.69	0.50
Potassium chloride	0.30	0.52	0.32
Magnesium sulfate heptahydrate	0.14	0.24	0.16
Trace minerals [6]	0.20	0.35	0.20
Special mix [7]	0.50	0.86	0.50
Choline chloride (25%)	0.75	1.30	0.88
DL-Methionine	0.19	0.52	
Lysine supplement [8]			0.51
L-Cystine			0.21
Glutamic acid			0.42
Glycine			0.08
Ethoxyquin [9]	0.0125	0.0216	0.0125

[1] Cerelose, Corn Products Company, New York.
[2] Solka Floc, Brown Company, Berlin, New Hampshire.
[3] Promine, Central Soya, Decatur, Indiana.
[4] Fermacto 500, The Borden Company, New York.
[5] Procter and Gamble Company, Cincinnati.
[6] Delamix with 2% zinc. Limestone Products Corporation of America, Newton, New Jersey. To provide in ppm: zinc, 40; iron, 40; copper, 4; manganese, 120; iodine, 2.4; and cobalt, 0.4.
[7] Vitamin mix provided the following per kilogram of diet: (IU) vitamin A, 6,600; vitamin D_3, 1,408; d-α-tocopheryl acetate, 22; (in milligrams) menadione sodium bisulfite, 4.4; thiamine·HCl, 6.8; riboflavin, 8.8; niacin, 52.9; Ca pantothenate, 22; pyridoxine·HCl, 6.6; folacin, 1.32; vitamin B_{12}, 0.022; biotin, 0.22; ascorbic acid, 22; procaine penicillin, 5.5; and coccidiostat (Amprol+, Merck & Company, Rahway, New Jersey), 500.
[8] Lyamine 50, Merck & Company, Rahway, New Jersey.
[9] Santoquin, Monsanto Company, St. Louis.

selected and distributed so that each pen had the same weight range and the same average body weight. In experiments 2 and 3, pairing of chicks by individual weight was not possible due to the large numbers of chicks used. Average pen weights and general weight ranges, however, were similar.

The same experimental diets, as shown in table 1, were used in all three experiments. These were a methionine-deficient basal (0.30%) diet and added levels of methionine to give an adequate methionine (0.55%) diet and an excess methionine (4.0%) diet. The diets were fed to three replicate groups of eight chicks from the different body composition groups in experiment 1.

In experiment 2, 9 pens of 10 chicks from each body composition group were fed the same level of methionine. The amount of food consumed was measured at 24-hour intervals. The chicks were killed at either 72, 144 or 288 hours. In experiment 3, the chicks were maintained at three different ambient temperatures. These were 11, 26–22 and 32°. After 24 hours, the chicks originally at 26° were lowered to 22°. Triplicate groups of 10 chicks were used in a $3 \times 3 \times 2$ factorial-type experimental design with three levels of methionine, three ambient temperatures and two different body composition groups. The chicks were fed the experimental diet for 360 hours.

When experiments 1 and 2 were terminated, half of the chicks from each pen

were killed by dislocating their necks without loss of blood. The carcasses were frozen, then cut into small portions and ground through a meat grinder. Aliquots were taken for moisture, and fat and protein determinations. Moistures were determined using the official AOAC method (8) for drying in vacuo at 95 to 100°. For fat analysis, the samples were first dried, then extracted in a Goldfisch fat extraction apparatus with anhydrous ether. Nitrogen determinations were made by the Kjeldahl method.

RESULTS AND DISCUSSION

In experiment 1, the group fed the low protein (6.4%) diet for 9 days during the preliminary period became excessively fat, averaging 24.1%. The chicks fed restricted amounts of the high protein (44%) diet had a carcass fat content of only 1.8%. Both groups had the same body weight. Selected chicks were then fed diets with different levels of methionine for 9 days. These treatments had a very marked effect on the body composition of the chicks killed at the end of the study, as shown in table 2. The carcass fat content increased in the nonobese birds and decreased in the obese chicks. Differences in body fat were present in both groups as the dietary methionine level changed. The lean chicks on the toxic methionine (4.0%) diet and the obese chicks on the methionine-deficient (0.30%) diet showed the smallest change in body fat from the initial percentages, from 1.8 to 6.5% and from 24.1 to 21.2% for the two groups, respectively. After 9 days on the adequate methionine diet there was little difference in the carcass fat content of the chicks. The fat content of the nonobese chicks had increased from 1.8 to 10.3%, whereas the obese chicks had decreased from 24.1 to 13.6%.

The voluntary food intake also is given in table 2. At each methionine level, the nonobese chicks consumed more than the obese chicks. The amount of food consumed per chick was 261 versus 27, 394 versus 189, and 185 versus 67 g for nonobese and obese chicks on the deficient, adequate and excess dietary methionine levels, respectively. This is in agreement with the observation of Huenemann et al.

TABLE 2

Effect of amino acid balance on weight gain, feed intake and body composition of obese and nonobese chicks [1] (exp. 1)

Observation	Initial body composition	Diet: Low methionine	Diet: Adequate methionine	Diet: Excess methionine
Avg wt gain, g				
Nonobese		124 ± 8.24 [2]	278 ± 5.43	54 ± 3.10
Obese		−38 ± 9.47	153 ± 6.40	19 ± 3.72
Feed consumed/chick, g				
Nonobese		261 ± 7.38	394 ± 2.15	185 ± 10.75
Obese		27 ± 7.79	189 ± 6.60	68 ± 6.32
		Body composition after fed diet for 9 days		
Ether extract, %				
Nonobese	1.8	9.6 ± 0.27	10.3 ± 0.15	6.5 ± 0.52
Obese	24.1	21.2 ± 1.51	13.6 ± 0.26	12.9 ± 1.79
Protein (N × 6.25), %				
Nonobese	18.9	17.9 ± 0.27	17.4 ± 0.16	18.5 ± 0.51
Obese	14.8	16.6 ± 0.64	16.5 ± 0.30	16.8 ± 0.19
Moisture, %				
Nonobese	76.2	69.2 ± 0.06	68.9 ± 0.09	71.9 ± 0.35
Obese	58.3	58.9 ± 1.65	66.3 ± 0.28	66.6 ± 1.51

[1] Three groups of eight male White Rock chicks used per treatment. One-half of the chicks were killed for carcass analysis.
[2] SE of mean.

(3) and Johnson et al. (1) who found that obese high school girls ate less than their normal weight controls.

Since the initial hunger of the nonobese chicks may have influenced the intake, which in turn may have affected the carcass composition, the study was repeated. To overcome this excessive initial hunger, the nonobese chicks were fed ad libitum for the last 3 days of the second preliminary period.

As in experiment 1, irrespective of ambient temperature in experiment 3 and at any dietary methionine level, the nonobese chicks consumed more food than their obese counterparts in experiments 2 and 3 (tables 3 and 4). Statistical analyses revealed that the differences in food consumption between the two body composition groups were highly significant ($P < 0.01$). Temperature in experiment 3, as well as methionine level in all three experiments, had a highly significant effect ($P < 0.01$) in controlling the amount of food consumed by either the obese or the nonobese chicks. The interaction between temperature and the methionine level in experiment 3 was also highly significant ($P < 0.01$).

Obese chicks on an adequate methionine diet tended to attain a normal body composition and food intake as time passed. This was accompanied by a gradual increase in body protein and a decrease in body moisture and fat, and consequently, they ate comparable amounts of food by the end of the study.

As the methionine level was either increased or decreased from the optimal level (0.55%), the chicks tended to consume less food. Chicks in a reduced ambient temperature and fed a methionine-deficient diet, however, increased their food consumption and consequently gained more than similar groups in a warmer temperature, regardless of initial body fat content (table 4).

TABLE 3

Effect of amino acid balance on voluntary food intake, of weight gain and carcass composition of obese and nonobese chicks (exp. 2)

Period	Low methionine diet		Adequate methionine diet		Excess methionine diet	
	Nonobese	Obese	Nonobese	Obese	Nonobese	Obese
days						
			Food consumption, g/chick			
0–3	66± 1.33 [1]	10±2.63	104±2.71	59±3.33	49± 0.91	25±3.15
0–6	129± 4.63	23±2.08	201±1.10	127±5.57	95± 1.56	54±2.13
0–12	234±11.4	41±2.61	452±5.89	358±6.29	187±11.05	117±2.38
			Body wt gain, g/chick			
0–3	26± 1.30	−9±1.77	76±2.24	62±3.79	14± 0.38	13±3.99
0–6	46± 1.54	−9±2.73	147±0.83	113±6.33	35± 3.17	24±1.31
0–12	81± 6.67	−13±1.97	334±2.60	310±5.15	61± 3.58	58±2.74
			Body ether extract, %			
0	3.0	25.1	3.0	25.1	3.0	25.1
3	6.3± 0.15	20.8±1.09	7.6±0.17	17.8±1.27	3.8± 0.38	17.9±0.76
6	6.2± 0.32	17.7±1.27	9.3±0.21	13.8±0.47	5.2± 0.21	12.8±1.07
12	6.8± 1.27	12.5±0.39	10.3±0.33	12.7±0.83	4.9± 0.74	7.8±0.22
			Body protein, %			
0	17.0	14.6	17.0	14.6	17.0	14.6
3	18.5± 0.27	16.3±0.39	17.9±0.21	15.6±0.40	19.0± 0.17	16.2±0.39
6	17.7± 0.28	16.1±0.22	17.8±0.55	17.3±0.14	18.4± 0.63	17.2±0.24
12	18.4± 0.32	16.3±0.19	18.0±0.10	17.2±0.21	18.9± 0.31	17.3±0.15
			Body moisture, %			
0	77.2	57.8	77.2	57.8	77.2	57.8
3	73.1± 0.20	61.4±0.97	72.7±0.62	64.7±0.61	75.2± 0.31	63.4±0.25
6	74.0± 0.44	64.2±1.51	70.8±0.67	67.4±0.60	74.1± 0.76	67.2±1.00
12	71.2± 1.01	67.1±0.43	68.8±0.29	67.2±0.57	73.2± 1.08	70.8±0.48

[1] SE of mean.

TABLE 4

Effect of amino acid balance and ambient temperature on voluntary food consumption and weight gain of obese and nonobese chicks [1] *(exp. 3)*

Temperature	Low methionine diet		Adequate methionine diet		Excess methionine diet	
	Nonobese	Obese	Nonobese	Obese	Nonobese	Obese
	Body wt gain, g/chick					
11°	151± 8.6 [2]	73±11.4	275±13.9	247± 5.6	56±8.9	53±12.7
22°	108± 8.6	12± 7.0	355± 1.6	367±11.0	80±3.5	91± 8.2
32°	93± 1.9	−7± 9.7	342±16.6	319± 8.7	84±6.2	82± 3.4
	Food consumption, g/chick					
11°	476± 7.9	242±49.7	547±11.9	454± 1.4	244±1.8	223±13.2
22°	283± 6.4	96±17.6	487± 4.9	466±15.7	242±3.9	184± 8.1
32°	271±11.7	64± 8.7	534±10.8	385±10.9	224±5.2	149± 2.7

[1] Triplicate groups of ten 20-day-old Arbor Acre male chicks.
[2] SE of mean.

This indicates that an energy need overcame the depressed food intake effected by the methionine-deficient diet. This is in agreement with the results reported by Klain et al. (5) and Harper and Rogers (6) of increased food consumption of rats fed imbalanced amino acid diets and exposed to a cold environment. Obese chicks fed a low methionine diet and maintained in a cold temperature of 11° were able to increase their food consumption of the imbalanced diet in contrast to similar groups in warmer temperatures (22 and 32°). This would suggest that caloric deficiency has a greater effect on the mechanism regulating food intake than body obesity or amino acid imbalance. However, by comparing food consumption of the obese chicks fed the low methionine diet in the reduced temperature (11°), with that of similar groups on an adequate methionine diet in a normal temperature (22°), it can be stated that the methionine level had a greater effect in controlling the amount of food consumed by the chicks than either temperature or body fat content. Nevertheless, both the depression in food consumption of obese chicks fed an adequate methionine diet and its continuous increase as they lose body fat towards a normal body composition, give support to a lipostatic, long-term regulatory mechanism.

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Proteolytic Activity and In Vitro Enzyme Stability in Small Intestinal Contents from Ruminants and Nonruminants at Different Ages [1,2]

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ABSTRACT Trypsin and chymotrypsin esterase activities and protein nitrogen were determined in digesta from the small intestine of calves and lambs fed liquid milk diets, from calves and sheep fed hay, and from rats and chickens fed concentrate diets. Total enzyme activities and in vitro protein digestion (digesta incubated for 2 hours at 37°) per unit body weight increased with both age and rumen development in calves. Total enzyme activities in lamb digesta were greater at 6 weeks than at 2 weeks of age, but were somewhat lower from yearling ruminating sheep, and in vitro protein digestion per unit body weight tended to decrease with age. Trypsin activity remained fairly constant in rat digesta from 1 to 9 months of age, but chymotrypsin activity declined. Lamb digesta had more enzyme activities per unit body weight than digesta from calves up to 41 days of age but less protein was digested in vitro. Except for 2-week-old lambs, digesta from the lower section of the intestine from all species had about 50% less protein, and less in vitro protein digestion than in digesta from the upper one or two sections. This indicated small amounts of readily digestible protein were present in the lower intestine. Less chymotrypsin than trypsin activity was retained during incubation of the digesta. Ratios of chymotrypsin-to-trypsin activities ranged from 0.6 to 0.8 in the bovine and ovine digesta, and 0.96 to 2.46 in the rat digesta.

The type and amount of protein entering the duodenum from the abomasum are altered when the rumen becomes functional. Duodenal contents contain mostly dietary protein before, and microbial protein after rumen development (1). Sineshchekov (2) reported a 25 to 50% decrease in the nitrogen content of chyme with increasing age of calves and change to solid feeds. But the amount of chyme and digestive juices, and rate of assimilation from the intestines increased three- to fourfold.

Digestive secretions have been reported to differ among breeds of cattle (2), and to increase with age in young calves (3) and lambs (4). But no comparisons of intestinal proteolytic enzymes have been made at various ages and among species.

In this experiment trypsin and chymotrypsin activities, and in vitro enzyme stability and protein digestion were determined in intestinal contents from calves, lambs, sheep, rats and chickens. The purpose of collecting these data was to compare: 1) the effect of age and rumen development, 2) ruminant versus nonruminant species, and 3) different sections of the small intestine.

MATERIALS AND METHODS

Animals and diets. Data were combined from 27 Holstein bull calves fed either whole milk, a milk substitute containing only milk protein or 86% of protein supplied by a trypsin inhibitor-free soy protein concentrate (5). The calves were killed at an average age of 10, 27 or 41 days. An additional 6 calves were fed whole milk at 10% of body weight to 7 weeks of age, concentrates and timothy hay to about 4 months of age and timothy hay ad libitum until killed at 5 months of age. Nine lambs (4 sets of twins and one single) were allowed to suckle their dams for about 15 minutes beginning at 8:00 AM, 12:00 noon and 4:30 PM. At other times lambs were

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[2] Taken in part from a thesis submitted by the senior author to the Graduate School of Michigan State University, in partial fulfillment of the requirements for the Ph.D. degree.

held in a pen with no access to hay or concentrate, to restrict rumen development. One of each set of twins was killed at from 12 to 14 days of age, and the remaining five lambs were killed at 6 weeks of age. Each lamb was weighed just before and after the 8:00 AM feeding on the day that it was killed, and three times the differences in body weights used as an estimate of daily milk intake. This might overestimate milk intake since it followed the longest time interval from the previous suckling. Four yearling wether sheep were fed reed canarygrass hay ad libitum for 3 to 4 weeks, prior to being killed 6 or 12 hours after feeding. Calves and lambs were killed from 1 to 1.5 hours after feeding. Rats weaned at 21 days of age were fed ad libitum either a milk or soy [3] protein diet (21% protein) with lactose or cerelose as a carbohydrate source until 28 or 42 days of age. Rats at 280 days and White Leghorn chickens at 210 days of age were fed ad libitum a cereal-soybean meal diet, with or without the addition of cycad husks or kernels.[4]

Intestinal contents. Digesta were obtained from the upper, middle and lower third of the small intestine from calves, lambs and sheep, as outlined by Gorrill and Thomas (5). Intestinal contents from 28- and 42-day-old rats were divided into only upper and lower halves, while those from rats and chickens killed at 280 and 210 days of age, respectively, were not divided into different sections. Volume and pH of the digesta from each section were recorded and aliquots of contents from the yearling sheep were centrifuged at 1300 $\times g$ for 30 minutes at 5° to remove solid food particles. Stability of trypsin and chymotrypsin and protein digestion in vitro were determined by incubating calf, lamb and sheep digesta at 37° for 2 hours. About an equal volume of glycerol was added to the digesta from 28- and 42-day-old rats, before incubation. Protein assays (Kjeldahl N $\times$ 6.25) were done on trichloroacetic acid precipitates (5) of calf and lamb digesta. The protein content of yearling sheep digesta was determined turbidimetrically as follows: 2 ml samples of diluted intestinal supernatant were mixed with 3 ml of 5% trichloroacetic acid or 3 ml of water. The samples were mixed, and after 30 seconds transmittances at 340 mμ corrected for the water blank were compared with bovine serum albumin as a standard (6). Trichloroacetic acid precipitates of 28- and 42-day-old rat intestinal contents were dissolved in 0.1 N NaOH and protein estimated as average values obtained by measuring absorbancy at 210 mμ (7) and difference absorbancy at 225 and 215 mμ (8).

Enzyme assays. Lamb, rat, chicken and 5-month-old calf intestinal contents were mixed with about an equal weight of glycerol to stabilize the enzymes (9) in nonincubated digesta, as well as in digesta after in vitro incubation. This value of glycerol was not realized when digesta from the younger calves and yearling sheep were obtained. The volume dilution of contents by glycerol was calculated by using specific gravities of 1.0 and 1.26 for the contents and glycerol, respectively. The supernatants of all samples were diluted in 0.15 M NaCl to obtain a suitable range of enzyme activities. Esterase activities of trypsin and chymotrypsin were assayed spectrophotometrically, using tosyl arginine methyl ester (TAME) and benzoyl tyrosine ethyl ester (BTEE) as substrates, respectively (10). Chymotrypsin assays on intestinal contents from calves fed whole milk or the all-milk replacer, and on those from the yearling sheep were determined with BTEE dissolved in 56% methanol (v/v). The BTEE was dissolved in 10% methanol (v/v), as described previously (10), for assays on digesta from the remaining calves, lambs, rats and chickens. Values obtained with 56% methanol were converted to those obtained with 10% methanol by multiplying the former by a factor of 2.2 (10). Enzyme activities were corrected for absorbance changes of blank reactions (TAME and BTEE solutions replaced by water and 56 or 10% methanol, respectively) when a nonenzymic apparent esterase activity (10) was evident in digesta from some calves. There was no evidence of substance(s) in lamb, sheep, rat or chicken digesta to interfere with the esterase enzyme assays.

[3] Promosoy, Central Soya, Decatur, Indiana.
[4] M. G. Yang and A. D. L. Gorrill 1967 unpublished data.

Statistical analyses. Analysis of variance was determined on data from the entire small intestine, and on each section separately for all species. Differences due to age, within species, and between species at comparable physiological ages, were determined by Duncan's multiple range test (11). Differences between sections of the small intestine, within species were determined by the *t* test (12).

RESULTS

Age of calves. Volume of digesta in the small intestine per unit body weight was greater in 5-month-old calves than in younger calves (table 1). Total activities of trypsin and chymotrypsin in the intestinal contents, based either on actual or physiological body size (wt $kg^{0.75}$), were about 10-fold higher in 5-month-old calves, than those in calves killed at 10 days of age. Calves killed at 27 or 41 days of age had about twice the total activities of these enzymes in the intestinal contents as those from 10-day-old calves, but the differences were not significant ($P < 0.05$). Total in vitro protein digestion (milligrams per 2 hours per kilogram body weight) was similar in digesta from calves killed at 10 and 27 days of age; this increased for 41-day-old calves, but remained nearly the same for 5-month-old calves (table 1). Approximately 35% of the protein in the samples was digested during the 2-hour incubation, regardless of age. In vitro protein digestion per unit of enzyme esterase activity, however, was about threefold greater in digesta from calves up to 41 days of age than in digesta from 5-month-old calves (not shown in table 1).

Age of lambs and sheep. The final body weights before suckling (table 1) of lambs

TABLE 1

Body weights, and physical, chemical and enzymic properties of small intestinal contents from calves, lambs, sheep, rats and chickens

Species and age	Body wt	Volume of intestinal contents [1]	Trypsin activity		Chymotrypsin activity		In vitro protein digested
	kg	*ml/kg body wt*	*units [2]/kg body wt*	*units/kg body $wt^{0.75}$*	*units [3]/kg body wt*	*units/kg body $wt^{0.75}$*	*mg/2 hr/kg body wt*
Bovine							
10 days (8) [4]	40.8	11.0 [a]	35.0 [A]	89 [A]	29.3 [A]	74 [A]	46.2 [a]
27 days (10)	48.7	13.9 [a]	72.5 [A]	191 [A]	40.4 [A]	107 [A]	43.5 [a]
41 days (9)	54.6	13.1 [a]	74.2 [A]	202 [A]	45.3 [A]	123 [A]	76.9 [b]
5 months (6)	128.6	19.4 [b]	378.0 [B]	1270 [B]	309.0 [B]	1032 [B]	72.8 [a,b]
Ovine							
13 days (4)	6.6	7.8 [a]	151.0 [a]	243 [a]	102.0 [a]	163 [a]	55.4
42 days (5)	12.9	8.4 [a]	437.0 [b]	832 [b]	231.0 [b]	437 [b]	27.9
1 year (4)	46.7	15.4 [b]	288.0 [a,b]	752 [b]	166.0 [a,b]	346 [b]	20.4
Rat							
28 days (4)	0.120	27.65 [a]	1241	732	1839 [A]	1066 [A]	4.6 [A]
42 days (11)	0.144	13.94 [b]	1297	786	895 [B]	544 [B]	52.4 [B]
280 days (16)	0.385		1391	1087			
Species comparisons							
Preruminants							
Calves (27)			62 [A]	164 [A]	39 [A]	102 [A]	55.5
Lambs (9)			310 [B]	570 [B]	173 [B]	315 [B]	40.1
Ruminants vs. nonruminants							
Calves (6)			378 [A]	1270 [a]	309 [A]	1032 [A]	72.8 [a]
Sheep (4)			288 [A]	752 [b]	166 [A]	346 [B,C]	20.4 [b]
Rats (31) [5]			1338 [B]	934 [b]	1146 [B]	684 [A,B]	—
Chickens (15)	1.98		51 [A]	59 [c]	—	—	—

[1] Means of a column within species and for species comparisons with different small or capital superscript letters are significantly different at $P < 0.05$ and < 0.01, respectively, using Duncan's multiple range test (11).
[2] One unit equals hydrolysis of 1 μmole TAME/minute.
[3] One unit equals hydrolysis of 1 μmole BTEE (4.7% methanol v/v in reaction mixture) per minute.
[4] Number of animals involved.
[5] Data on chymotrypsin activity are from 15 rats.

killed at 2 and 6 weeks of age were 49 and 210% greater than the average birth weight of 4.3 kg, and daily body weight gains from birth to slaughter were 169 and 217 g, respectively. Estimated milk intake for one feeding before slaughter averaged 9.1 (600 g) and 5.6% (720 g) of live body weight at 2 and 6 weeks of age, respectively.

Yearling sheep had a larger volume of intestinal contents per unit body weight than the lambs (table 1). Total activities of intestinal trypsin and chymotrypsin per unit of actual or physiological body weight were two- to threefold higher in lambs killed at 6 weeks, than in those killed at 2 weeks of age. In contrast to calves, however, yearling sheep tended to have less total activities of these enzymes per unit body size in the intestinal contents than those found in 6-week-old lambs. Protein content (not shown in table 1) and total in vitro protein digestion (table 1) both tended to decline ($P > 0.05$) with increasing age in the ovine species, which was opposite to that previously noted in the bovine. From 30 to 50% of the protein was digested during the incubation of ovine digesta, except for average values of 18 and 4%, respectively, in lower intestinal contents for lambs killed at 6 weeks and for yearling sheep.

Age of rats. Volume and chymotrypsin activity of intestinal contents per unit of body weight were both less in rats at 42 days of age than in those at 28 days of age (table 1). Although trypsin activity changed little with age, in vitro protein digestion was 11-fold greater in digesta from the older rats.

Species comparisons. Data in table 1 were arranged to compare: 1) milk-fed calves and lambs with nonfunctional rumens, and 2) ruminants versus monogastric animals fed hay and concentrate diets, respectively. Lambs had about fivefold higher total activities of intestinal trypsin and chymotrypsin per unit body weight than those in calves of comparable ages. The opposite difference, however, was evident between older calves and sheep fed grass hay. Rat intestinal contents contained the highest activities of these enzymes, when based on actual body weight, but were less than those in digesta from calves when based on metabolic body weight. Intestinal contents from chickens had low trypsin esterase activity.

Total in vitro protein digestion was greater in digesta from calves than in those from sheep (table 1). The amount of protein digested per unit of enzyme activity was much greater, and enzyme activity per unit of protein was much less in digesta from calves (table 2). Protein digestion per unit of enzyme activity was also low in rat digesta, but could not be statistically compared with calf and sheep data for two reasons: 1) the rat intestine was divided into only two sections, and more important, 2) the digesta were incubated with glycerol which might change enzyme activity and increase enzyme stability. Rat digesta did, however, contain the highest concentrations of protein. Trypsin was less stable during in vitro incubation of upper intestinal contents from the bovine than the ovine, but the opposite species difference occurred for chymotrypsin in digesta from the lower intestine (table 2). Ratios of intestinal chymotrypsin-to-trypsin activities averaged from 0.60 to 0.80 for the ruminants, but from 0.96 to 2.46 for the rat.

Intestinal segments. Digesta from the upper sections of the small intestine contained more protein, and protein digested in vitro (milligram per milliliter) and per unit of enzyme activity were greater than in digesta from the lower section (table 2). No in vitro protein digestion occurred in rat lower intestinal contents. Enzyme activities per unit of protein were highest, and enzyme stability during incubation was least, in contents from the lower section. Chymotrypsin-to-trypsin ratios were similar in digesta from the three sections of the small intestine from the ruminants, but this ratio was smaller in lower than that in upper intestinal contents from rats.

In vitro stability of trypsin was always greater than that for chymotrypsin in digesta from all sections of the small intestine.

DISCUSSION

The proteolytic digestive function of the calf intestine relative to body weight increased with both age and rumen development, particularly for total esterase ac-

TABLE 2

Physical, chemical and enzymic properties of digesta from different segments of the small intestine from calves, lambs, sheep and rats

Criteria	Species	Intestinal segment			
		Upper	Middle	Lower	*t* test
Protein digested/trypsin unit, mg	bovine	1.91 [A 1]	1.02 [A]	0.80 [A]	U>>M,L [2]
	ovine	0.22 [B]	0.14 [B]	0.06 [B]	U>L
	rat	0.41		0.01	U>L
Protein digested/chymotrypsin unit, mg	bovine	1.72 [A]	1.86 [A]	1.26 [A]	ns [3]
	ovine	0.48 [B]	0.17 [B]	0.10 [B]	U>M,L
	rat	0.20		0.02	U>L
Trypsin units/mg protein	bovine	0.47 [A]	0.89 [A]	1.51 [A]	M>U,L>>>U
	ovine	3.70 [B]	7.26 [B]	7.37 [B]	ns
	rat	1.36		6.34	L>U
Chymotrypsin units/mg protein	bovine	0.41 [A]	0.63 [A]	0.97 [A]	M,L>U
	ovine	1.63 [B]	3.53 [B]	4.00 [B]	ns
	rat	1.99		8.19	L>>U
Protein concn, mg/ml	bovine	17.4 [a]	17.2	8.9	U,M>>>L
	ovine	11.5 [b]	14.6	7.7	ns
	rat	38.6		19.5	ns
Protein digested, mg/ml	bovine	5.89	5.58	2.19	U,M>>>L
	ovine	4.85	5.75	2.02	M>L
	rat	8.01		0.06	U>L
Trypsin in vitro stability [4]	bovine	0.82 [A]	0.80	0.75	ns
	ovine	0.98 [B]	0.80	0.68	U>>L
	rat	0.97		0.98	ns
Chymotrypsin in vitro stability [4]	bovine	0.56	0.54	0.43 [a]	ns
	ovine	0.72	0.57	0.27 [b]	U>>>L,M>>L
	rat	0.87		0.80	ns
Ratio: chymotrypsin to trypsin	bovine	0.80 [a]	0.70	0.79	ns
	ovine	0.60 [b]	0.72	0.61	ns
	rat	2.46		0.96	U>L
Total trypsin units [5]	bovine	3506	5250	2570	M>>>U,L
	ovine	2096	3086	1473	M>L
	rat	34		123	L>>>U
Total chymotrypsin units [6]	bovine	2914	3931	1780	U>L,M>>>L
	ovine	1288	1733	717	M>>L
	rat	55		108	L>U

[1] Means with different small or capital superscript letters are significantly different at $P < 0.05$ and < 0.01, respectively.
[2] > Difference significant at $P < 0.05$; >> Difference significant at $P < 0.01$; >>> Difference significant at $P < 0.001$.
[3] Difference between means significant at $P > 0.05$.
[4] Ratio of enzyme activity of incubated-to-nonincubated intestinal contents.
[5] See footnote 2, table 1.
[6] See footnote 3, table 1.

tivities of trypsin and chymotrypsin. However, in vitro protein digestion per unit of enzyme activity was least in digesta from calves with functional rumens. Secretion of proteolytic enzymes in pancreatic juice of calves also tended to increase with age (3). Improved digestibility of soybean protein by calves at 26 to 28 days of age, compared with that at 10 to 14 days of age, has also been reported (13).

Total activities of intestinal trypsin and chymotrypsin per unit of body weight increased with age in preruminant lambs, which confirmed data on total proteolytic activity with casein at pH 8.5 (4). But, in contrast to the bovine, there was no increase in mature sheep with functional rumens in this study. There was also a tendency for in vitro protein digestion per unit body weight to decline with advancing

age and rumen development in sheep. The yearling sheep were killed 6 or 12 hours after feeding, whereas the lambs and calves were killed 1 to 1.5 hours after feeding. The functioning rumen, however, provides a continuous flow of digesta to the duodenum (2), and there were no significant differences between data from sheep killed 6 or 12 hours after feeding in this study. Intestinal trypsin activity was nearly constant in rats from 1 to 9 months of age, whereas chymotrypsin activity decreased with age.

Lamb intestinal contents contained more total trypsin and chymotrypsin activities per unit body weight than did those in calves to 41 days of age, but the differences were not as great when based on metabolic body size. The differences in these digestive enzymes may be associated in some way with the relative growth rates and feed intake of the young lamb and calf, and protein content of the diets. Lambs increased their birth weight threefold, from birth to 6 weeks of age. Estimated daily intake of milk at 2 and 6 weeks of age was 27 and 17% of body weight, respectively. The body weight of calves at 6 weeks of age and fed milk at 10% of body weight daily (table 1) was only about 1.40 times greater than birth weight. The average protein content of cow's and ewe's milk has been reported to be 3.1 and 5.8%, respectively (14). Rats contained somewhat less intestinal proteolytic enzymes than ruminating calves when based on metabolic body size. Chickens had very low levels of intestinal trypsin.

Despite the greater proteolytic enzyme activities in lamb intestinal contents, total in vitro protein digestion and protein digested per unit of enzyme activity was low compared with values in calf intestinal contents. Intact protein in the digesta would not appear to be a limiting factor in the case of lambs, since only about 30 to 50% of the total protein was digested. The remaining protein, however, may be largely of endogenous origin and more resistant to digestion (15). Furthermore, as previously discussed (5), protein digestion under these in vitro conditions may not adequately simulate conditions in vivo. Thus, differences in extent of either exogenous protein digestion, or ratio of exogenous to less digestible endogenous protein, or both, may be operative in the observations made.

Amounts of protein digested in vitro and enzymatic efficiency of protein digestion (protein digested per unit of trypsin or chymotrypsin activity) were greater in contents from the upper sections of the small intestine than in those from the lower section. In contrast to calves and sheep, lower intestinal contents from rats contained more trypsin and chymotrypsin activities than did those from the upper intestine, which confirmed previous data with rats (16). Nearly complete digestion and absorption of dietary protein was shown to occur in the distal duodenum and proximal jejunum in nonruminants (15), leaving low levels of readily digestible protein in digesta from the lower small intestine. The presence of relatively large amounts of rumen microbes in the small intestine of ruminants, however, could alter the ratios of exogenous-to-endogenous protein. The greater loss of enzyme activity during incubation of digesta from the lower than from the upper or middle sections of the small intestine in this study, and reported previously in rats (17), may be attributed to one or more of the following factors in lower intestinal contents: 1) self-digestion of proteolytic enzymes due to either small amounts of dietary protein (18), or lower calcium levels (19, 20), or both, in the digesta; 2) destruction of enzymes by microbial proteases (21); and 3) the amount and type of protein entering the duodenum. To the extent that digestive enzymes are degraded in the lower small intestine, this process would allow reabsorption of any liberated amino acids, and minimize excessive losses of endogenous protein in the feces. However, low values for protein digested in contents from the lower small intestine and the comparatively high activities of trypsin and chymotrypsin (units per milliliter of contents or units per milligram protein) indicate that these enzymes have not been extensively degraded in this portion of the intestine.

Greater stability of trypsin than chymotrypsin during in vitro incubation of calf, rat, lamb and sheep intestinal contents confirms other data with rat digesta (16, 18), but in human intestinal juice chymotrypsin was more stable than trypsin (22).

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Chemical Pathology of Acute Amino Acid Deficiencies: Morphologic and Biochemical Changes in Young Rats Force-fed a Threonine-deficient Diet [1]

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ABSTRACT To observe the relative importance of partial versus complete absence of threonine in inducing a nutritional imbalance, young rats of the Sprague-Dawley strain were force-fed a threonine-deficient diet, one containing 0.25 or 0.5% instead of 1% DL-threonine, for 3, 7, 10, 14, 21 and 28 days. Animals force-fed the threonine-deficient diets gained less weight than control animals fed the complete diet, developed atrophy of the pancreas, submaxillary gland, parotid, stomach and thymus, and showed evidence of increased radioactive amino acid incorporation into hepatic and plasma protein, but decreased incorporation into gastrocnemius muscle protein. The results indicate that the level of dietary threonine plays an important role in the pathologic changes in the liver caused by threonine deficiency.

In earlier studies (1–3) we found that young rats force-fed purified diets devoid of single essential amino acids developed pathologic changes that closely resembled many of those found in infants with kwashiorkor (4). In these studies animals were force-fed for 3, 7 or 14 days. Longer durations were not studied since most animals force-fed the experimental diets, especially one devoid in threonine, died within 7 to 14 days.

The purpose of the present study was to determine whether young rats force-fed a diet containing threonine but at less than an optimal level (0.25 or 0.5% instead of 1.0% DL-threonine) could be studied for longer durations and to observe the pathologic changes induced by the deficient diet. It was of interest to learn whether the organ changes, both biochemical and structural, would be similar to those observed in earlier studies with a threonine-devoid diet. The results indicate that young rats force-fed a threonine-deficient diet for up to 4 weeks tolerate the diet well, gain much less weight than do control animals fed the complete diet, develop pathologic changes such as atrophy of the pancreas, submaxillary gland, parotid, stomach and thymus, and show evidence of increased protein synthesis in the liver and decreased protein synthesis in gastrocnemius muscle.

METHODS

Male and female rats of the Sprague-Dawley strain,[2] 1 month old, weighing on the average 70 g, were used. The animals were maintained on a commercial diet [3] for at least 4 days before the experiments were begun. In all experiments groups of animals, each of the same sex, age and weight were used.

The basal diet was similar to that used in our earlier experiments (1–3, 5–8). The percentage of added components was as follows: essential amino acids, 9.2; nonessential amino acids, 8.1; salt mixture, 4; vitamin-sucrose or vitamin-casein mixture, 5; corn oil,[4] 5; cod liver oil, 1.5; and sucrose or dextrin, 67.2. In experiment 89, the experimental diet contained 67.2% sucrose and 5% vitamin-casein mixture (9), which contributed 0.23% threonine to the diet mixture. The complete diet contained in addition 1% DL-threonine. In experiments 383 and 460, the diets contained 67.2% dextrin and 5% vitamin-sucrose mixture (1). In the latter two experiments,

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[2] Sprague-Dawley, Inc., Madison, Wisconsin.
[3] Wayne Lab-Blox, Allied Mills, Inc., Chicago.
[4] Mazola, Corn Products Company, New York.

0.25% or 0.50% DL-threonine was added to the diets given the experimental animals and 1.0% was added to the control diets. Dextrin was used to compensate for the differences in threonine in the experimental groups. All diets were blended with distilled water such that each milliliter of diet mixture contained 0.5 g of diet and was in a suitable form for administration by stomach tube.

Rats were force-fed with plastic tubes. The diets were administered twice daily, at 8:30 AM and 5:30 PM. The animals received an average daily feeding of 0.9 g of diet per 10 g of initial body weight. The complete diet was force-fed to the control rats throughout and to all experimental animals for several days prior to beginning the deficient diets. All rats had free access to water. Rats were housed in individual wire cages with raised bottoms and kept in an air-conditioned room. After being force-fed the experimental diets for 3, 7, 10, 14, 21 or 28 days, rats were anesthetized with ether and exsanguinated the following morning, approximately 18 hours after the last feeding. In two experiments some animals were fed one diet for a certain length of time and then were switched to another diet as follows: in experiment 383, three rats fed the 0.5% threonine-deficient diet for 27 days were then fed a threonine-devoid diet for 4 days; in experiment 460, four rats fed the complete diet for 25 days were then fed the threonine-devoid diet for 3 days.

Rats were weighed at the beginning, at regular 3- or 4-day intervals and at the end of each experiment. In experiments 383 and 460, each animal received an intraperitoneal injection of an aqueous solution of 2.5 μCi (7.9 μCi/μmole) and 2.5 to 5 μCi (10 μCi/μmole), respectively, of ^{14}C-leucine, uniformly labeled, 1 hour before killing. In experiment 460, one group of animals received ^{14}C-leucine 5 minutes before killing. The organs were weighed fresh. In paired organs, the right organ was weighed. Pieces of tissue from selected organs were fixed in Zenker-formol solution and in 10% formalin. Paraffin sections routinely were stained with hematoxylin and eosin. Frozen sections of formalin-fixed liver were stained with oil red O. The methods used for chemical analyses have been described in detail in earlier studies (3, 5–7). Radioactivity in protein was measured using a liquid scintillation spectrometer.[5] In experiment 460, the levels of free amino acids of pooled livers of one group of control and experimental animals were determined in an amino acid analyzer,[6] and the specific activity of the free leucine in the liver was determined after measuring its radioactivity in a liquid scintillation spectrometer.[7]

RESULTS

In figure 1 (A, B and C) the changes in body weight are presented for experiments 89, 383 and 460. Even though the quantity of food fed to all animals was the same in each experiment, rats force-fed the complete diet gained on the average of 1.7 g/day; rats fed the 0.5% threonine-deficient diet gained 0.9 g/day; and rats fed the 0.25% threonine-deficient diet gained 0.3 g/day. In experiment 89 the control animals gained more weight per day than the controls of the other two experiments (fig. 1). This greater weight gain was most probably due to the higher percentage of amino acids in the diet, 23.3 rather than 17.3%, due to the addition of 5% casein.

In table 1 the weights of the liver, spleen, kidney and gastrocnemius muscle are summarized. Since the body weights of the animals were different depending on the duration of the feedings, the organ weights have been expressed per 100 g terminal body weight. Regardless of whether the animals were fed for 3 days or up to 31 days, the liver weights were greater in the rats fed the 0.25% threonine-deficient diet than their comparable controls fed the complete diet. Rats fed the 0.5% threonine-deficient diet showed increases in liver weights after 3 and 4 weeks. Gastrocnemius muscle and spleen weights were decreased, whereas kidney weights were increased, in rats fed the 0.25% threonine-deficient diet for 2 to 4 weeks. These organs, however, showed little change in weight in rats fed the 0.5% threonine-deficient diet in comparison with controls.

[5] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Illinois.
[6] Spinco, Model no. 120 B, Beckman Instruments, Inc., Fullerton, California.
[7] Nuclear-Chicago Corporation, Des Plaines, Illinois.

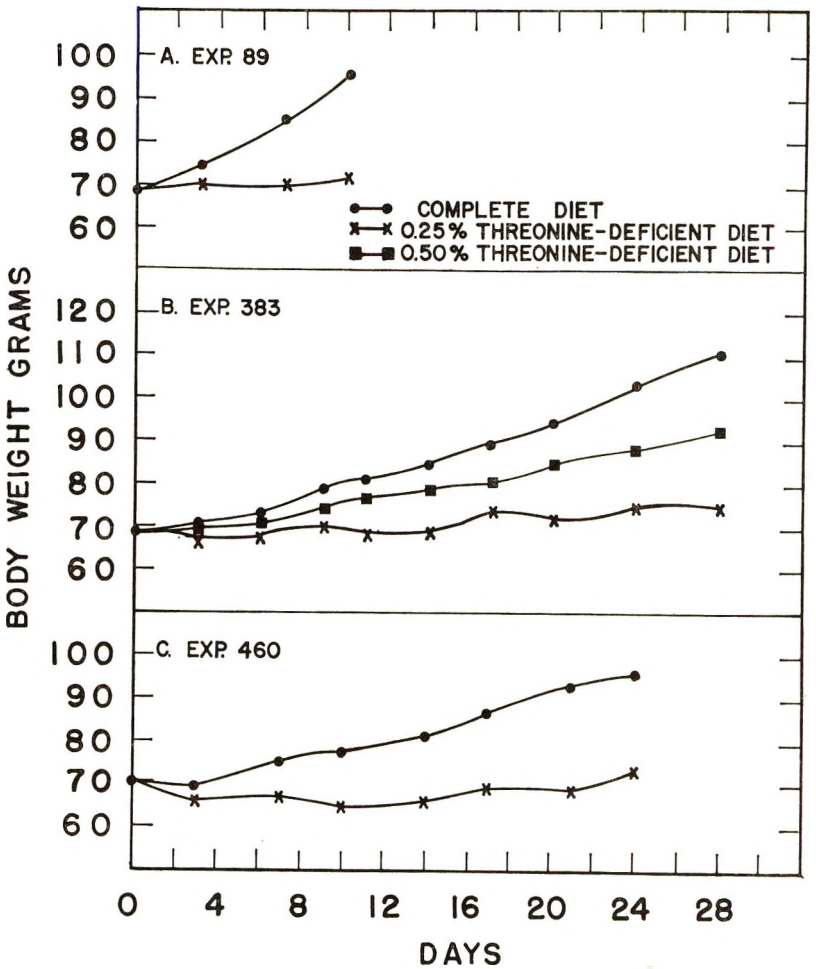

Fig. 1 Changes in body weight.

Table 2 summarizes analyses of hepatic lipid, glycogen and protein, as well as protein of spleen, kidney, gastrocnemius muscle and plasma. Rats fed the 0.25% threonine-deficient diet showed small increases in hepatic lipids in most cases beginning at day 7 and continuing up to day 28. Liver glycogen was increased in rats fed the 0.25% threonine-deficient diet for 3 to 10 days in experiment 89, but not in the experimental groups of longer duration in experiments 383 and 460. Hepatic protein showed little change, or a small increase, in rats fed the 0.25% threonine-deficient diet. Rats fed the 0.5% threonine-deficient diet in experiment 383 showed little changes in hepatic lipid, glycogen and protein, as well as in protein content of the spleen, kidney and gastrocnemius muscle. In rats fed the 0.25% threonine-deficient diet the protein content decreased in spleen, gastrocnemius muscle and plasma but not in kidney.

Table 3 shows the results of ^{14}C-leucine incorporation into protein of liver, spleen, kidney, gastrocnemius muscle and plasma of rats force-fed the control and experimental diets for 14 to 31 days. The results are expressed as specific activity (radioactivity per milligram protein) as well as per total organ corrected per 100 g terminal body weight. The results indicate that both the specific activity and the total protein radioactivity of the livers, as well as

TABLE 1

Body and organ weights of rats force-fed complete or threonine-deficient diet for 3 to 31 days

Exp. no.	Group [1]	Duration	No. of rats	Sacrifice wt [2]	Liver	Spleen	Kidney	Gastrocnemius muscle
		days		*g*	*g/organ/100 g body wt* [3]			
89	C	3	8	74	3.85 ± 0.03			
	TD ¼		9	70	4.48 ± 0.21 [6]			
	C	7	8	85	3.96 ± 0.12			
	TD ¼		8	70	5.17 ± 0.18 [6]			
	C	10	6	96	3.72 ± 0.07			
	TD ¼		6	72	5.57 ± 0.34 [6]			
383	C	14	4	85	4.12 ± 0.11	0.298 ± 0.003	0.453 ± 0.020	0.580 ± 0.016
	TD ¼		4	69	5.15 ± 0.05 [6]	0.173 ± 0.027 [6]	0.550 ± 0.014 [6]	0.465 ± 0.030 [7]
	TD ½		4	79	4.13 ± 0.03	0.239 ± 0.009 [6]	0.500 ± 0.010	0.577 ± 0.005
	C	21	4	95	3.83 ± 0.11	0.249 ± 0.020	0.448 ± 0.006	0.586 ± 0.020
	TD ¼		4	72	4.86 ± 0.11 [6]	0.215 ± 0.018	0.531 ± 0.012 [6]	0.525 ± 0.015
	TD ½		4	85	4.63 ± 0.31	0.283 ± 0.008	0.492 ± 0.019	0.577 ± 0.010
	C	28	3	111	3.67 ± 0.21	0.263 ± 0.025	0.402 ± 0.022	0.624 ± 0.030
	TD ¼		4	75	5.56 ± 0.12 [6]	0.172 ± 0.011 [7]	0.551 ± 0.027 [6]	0.567 ± 0.024
	TD ½		4	93	4.54 ± 0.26 [7]	0.278 ± 0.031	0.444 ± 0.029	0.625 ± 0.018
	C	31	3	119	3.32 ± 0.08	0.262 ± 0.009	0.394 ± 0.018	0.634 ± 0.028
	TD ½		3	97	3.83 ± 0.11 [7]	0.268 ± 0.008	0.432 ± 0.022	0.633 ± 0.030
	TD ½ → TD [4]		3	89	4.59 ± 0.28 [7]	0.181 ± 0.008 [6]	0.497 ± 0.019 [7]	0.620 ± 0.036
460	C	14	4	77	3.83 ± 0.06	0.276 ± 0.018	0.449 ± 0.010	0.581 ± 0.009
	TD ¼		5	58	4.95 ± 0.22 [6]	0.196 ± 0.014 [6]	0.573 ± 0.014 [6]	0.487 ± 0.029 [7]
	C	21	3	99	4.06 ± 0.37	0.253 ± 0.020	0.453 ± 0.024	0.652 ± 0.026
	TD ¼		6	64	5.15 ± 0.17 [7]	0.191 ± 0.015 [7]	0.570 ± 0.017 [6]	0.510 ± 0.014 [6]
	S	—	3	62	3.62 ± 0.10	0.383 ± 0.035 [7]	0.503 ± 0.011	0.562 ± 0.016 [7]
	C	28	5	102	3.68 ± 0.08	0.265 ± 0.013	0.447 ± 0.007	0.678 ± 0.039
	TD ¼		7	76	4.94 ± 0.14 [6]	0.219 ± 0.004 [6]	0.541 ± 0.018 [6]	0.567 ± 0.016 [7]
	C → TD [5]		4	92	4.25 ± 0.09 [6]	0.219 ± 0.013 [7]	0.481 ± 0.014	0.668 ± 0.012

[1] C indicates complete diet; TD ¼ indicates threonine-deficient diet containing 0.23 or 0.25% threonine; TD ½ indicates threonine-deficient diet containing 0.5% threonine; TD indicates threonine-devoid diet; S indicates stock diet.
[2] Average onset weights were for experiment 89, 70 g; for experiment 383, 69 g; and for experiment 460, 70 g.
[3] Values expressed as mean ± SE of mean.
[4] Animals were fed the threonine-deficient diet containing 0.5% threonine for 27 days and then changed to threonine-devoid diet for 4 days.
[5] Animals were fed the complete diet for 25 days and then changed to the threonine-devoid diet for 3 days.
[6] $P < 0.01$.
[7] $0.05 > P < 0.01$.

TABLE 2

Analysis of liver, spleen, kidney, gastrocnemius muscle and plasma of rats force-fed complete or threonine-deficient diet for 3 to 31 days

Exp. no.	Group [1]	Duration	No. of rats	Liver			Spleen protein [2]	Kidney protein [2]	Gastrocnemius muscle protein [2]	Plasma protein
				Total lipid	Glycogen	Protein				
		days		*mg/organ/100 g body wt*						*g/100 ml*
89	C	3	8	216 ± 36 [3]	19 ± 6 [3]	716 ± 28 [3]				
	TD ¼		9	238 ± 17	79 ± 18 [6]	683 ± 19				
	C	7	8	228 ± 19	52 ± 25	714 ± 18				
	TD ¼		8	333 ± 23 [6]	87 ± 29	760 ± 28				
	C	10	6	216 ± 10	39 ± 14	563 ± 28				
	TD ¼		6	386 ± 50 [6]	68 ± 28	818 ± 35 [6]				
383	C	14	4	191 ± 1	140 ± 25	935 ± 42	59	83	82	
	TD ¼		4	263 ± 15 [6]	134 ± 19	1190 ± 43 [6]	31	97	48	
	TD ½		4	215 ± 19	139 ± 21	923 ± 16	50	102	84	
	C	21	4	173 ± 5	158 ± 30	910 ± 27	46	87	65	
	TD ¼		4	228 ± 15 [7]	133 ± 17	1104 ± 50 [7]	43	100	73	
	TD ½		4	210 ± 13 [7]	285 ± 85	1120 ± 100	52	86	70	
	C	28	3	186 ± 16	189 ± 23	884 ± 50	47	78	119	
	TD ¼		4	297 ± 23 [7]	203 ± 25	1196 ± 29 [6]	31	101	86	
	TD ½		4	255 ± 37	257 ± 29	1114 ± 115	50	87	109	
	C	31	3	185 ± 8	135 ± 47	785 ± 28	48	80	86	
	TD ¼		3	218 ± 15	181 ± 11	920 ± 34 [7]	50	79	60	
	TD ½ → TD [4]		3	356 ± 53 [7]	166 ± 20	1064 ± 82 [7]	36	80	79	
460	C	14	4	210 ± 30	57 ± 14	1091 ± 16	47	87	120	5.9 ± 0.18 [3]
	TD ¼		5	238 ± 12	30 ± 8	1193 ± 61	36	98	80	4.8 ± 0.39 [7]
	C	21	3	184 ± 4	108 ± 9	1001 ± 105	44	84	124	6.2 ± 0.59
	TD ¼		6	267 ± 19 [6]	104 ± 32	1161 ± 59	34	122	85	4.8 ± 0.19
	S	—	3	278 ± 23 [7]	2 ± 2 [6]	889 ± 1	72	104	125	5.9 ± 0.19
	C	28	5	170 ± 1	84 ± 12	955 ± 42	56	98	129	6.4 ± 0.25
	TD ¼		7	207 ± 13 [7]	107 ± 10	1127 ± 37 [7]	43	116	89	6.2 ± 0.36
	C → TD [5]		4	211 ± 10 [6]	69 ± 18	1077 ± 13	47	105	151	5.4 ± 0.31 [7]

[1] C indicates complete diet; TD ¼ indicates threonine-deficient diet containing 0.23 or 0.25% threonine; TD ½ indicates threonine-deficient diet containing 0.50% threonine; TD indicates threonine-devoid diet; S indicates stock diet.
[2] Values based on determination of pooled specimens.
[3] Mean ± SE of mean.
[4] Animals were fed the threonine-deficient diet containing 0.5% threonine for 27 days and then changed to the threonine-devoid diet for 4 days.
[5] Animals were fed the complete diet for 25 days and then changed to the threonine-devoid diet for 3 days.
[6] $P < 0.01$.
[7] $0.05 > P < 0.01$.

TABLE 3

Incorporation of ^{14}C-leucine into protein of liver, spleen, kidney, gastrocnemius muscle and plasma of rats force-fed complete or threonine-deficient diet for 14 to 31 days

Exp. no.	Group [1]	Duration	No. of rats	Liver protein		Spleen protein [2]		Kidney protein [2]		Gastrocnemius protein [2]		Plasma protein [2]
		days		S.A.[3]	*cpm/liver/ 100 g body wt × 10^{-3}*	S.A.[3]	*cpm/ spleen/ 100 g body wt × 10^{-3}*	S.A.[3]	*cpm/ kidney/ 100 g body wt × 10^{-3}*	S.A.[3]	*cpm/ muscle/ 100 g body wt × 10^{-3}*	S.A.[3]
383	(1) C	14	4	565 ± 27 [4]	525 ± 11 [4]	605	35.5	426	35.4	97	8.0	834
	(2) TD ¼		4	837 ± 53 [9]	991 ± 54 [9]	582	18.1	615	59.6	106	5.1	1291
	(3) TD ½		4	717 ± 38 [10]	663 ± 46 [10]	762	37.8	500	51.2	120	10.1	1050
	(4) C	21	4	370 ± 60	342 ± 64	336	15.3	283	24.6	77	4.9	533
	(5) TD ¼		4	550 ± 56	608 ± 72 [10]	444	18.9	451	45.2	104	7.6	738
	(6) TD ½		4	545 ± 79	622 ± 125	523	27.0	336	28.9	65	4.6	783
	(7) C	28	3	574 ± 43	509 ± 58	593	28.0	424	33.2	87	10.3	749
	(8) TD ¼		4	841 ± 88	1010 ± 122 [10]	545	17.0	546	55.0	101	8.7	1113
	(9) TD ½		4	668 ± 106	734 ± 110	536	26.6	496	43.2	90	9.8	887
	(10) C	31	3	434 ± 40	338 ± 23	478	23.0	372	29.7	78	6.8	543
	(11) TD ½		3	633 ± 45 [10]	584 ± 59 [10]	644	32.5	502	39.4	99	6.0	752
	(12) TD ½ → TD [5]		3	897 ± 139 [10]	975 ± 230	502	17.8	533	42.6	67	5.3	1136
460	C	14	4	809 ± 98	887 ± 117	915	43.2	1015	88.3	132	15.8	952
	TD ¼		5	1105 ± 102	1320 ± 145	867	30.9	630	61.7	122	9.8	1639
	C [6]	21	3	1002 ± 156	975 ± 96	782	34.3	657	55.1	146	18.1	1381
	TD ¼ [8]		3	1753 ± 19 [9]	1943 ± 168 [9]	1089	38.4	925	112.9	182	19.3	2068
	S	—	3	758 ± 46	673 ± 46	529	37.9	461	47.9	95	11.9	441
	TD ¼	21	3	982 ± 188	1171 ± 177	522	17.0	511	61.8	75	4.8	1184
	C [7]	28	3	753 ± 32	699 ± 44	542	15.9	489	48.9	88	11.4	937
	TD ¼ [7]		5	1081 ± 52 [9]	1107 ± 59 [9]	670	28.9	574	66.6	83	7.4	1187
	C → TD [7,8]		4	980 ± 147	1048 ± 152	452	21.2	510	53.6	65	9.8	1298

[1] C indicates complete diet; TD ¼ indicates threonine-deficient diet containing 0.25% threonine; TD ½ indicates threonine-deficient diet containing 0.5% threonine; TD indicates threonine-devoid diet; S indicates stock diet. All animals received 2.5 μCi ^{14}C-leucine 1 hour before killing unless otherwise indicated.
[2] Values based on determinations of pooled specimens.
[3] S.A. = specific activity (cpm/mg protein).
[4] Mean ± SE of mean.
[5] Animals were fed the threonine-deficient diet containing 0.5% threonine for 27 days and then changed to the threonine-devoid diet for 4 days.
[6] Animals received 5 μCi ^{14}C-leucine 1 hour before killing.
[7] Animals received 3 μCi ^{14}C-leucine 1 hour before killing.
[8] Animals were fed the complete diet for 25 days and then changed to the threonine-devoid diet for 3 days.
[9] $P < 0.01$.
[10] $0.05 > P < 0.01$.

the specific activity of plasma proteins of all experimental groups, were increased over those of the comparable controls. In animals fed the 0.25% threonine-deficient diet total protein radioactivity in gastrocnemius muscle and spleen was usually decreased in comparison with controls. Incorporation into kidney protein was increased in many of the experimental groups in comparison with controls.

Since the control and experimental animals after 2-, 3- and 4-week intervals each received the same quantity of ^{14}C-leucine prior to killing, even though there were differences in body weight (table 1 and fig. 1), it was necessary to consider whether the differences in incorporation into organ proteins between control and experimental animals could be due to alterations in pool size of free leucine. To obtain an indication of the specific activity of the precursor free amino acid close to the time of incorporation, the total nonprotein acid-soluble radioactivity was determined at the time of killing. Since this value gives a rough index of the pool size of the labeled precursor amino acid which influences the extent of incorporation into protein, it was used to obtain a ratio (total radioactivity in organ protein to total radioactivity in acid-soluble fraction of total organ) which gives a relative expression of the extent of incorporation of isotope into organ protein. These corrected values only introduced minor differences from the total radioactivity values expressed in table 3. In addition, from the results summarized in table 3 it is possible to make a few relative comparisons to rule out that the differences could have been due to altered pool sizes of ^{14}C-leucine. In experiment 383, since all animals received 2.5 μCi ^{14}C-leucine, one can compare animals according to terminal body weights (table 3). Groups (1) C, (3) TD ½, (6) TD ½ with terminal body weights 85, 79 and 85 g, respectively, and groups (4) C, (9) TD ½ and (11) TD ½ with terminal body weights of 95, 93 and 97 g, respectively, revealed in each case differences in specific activity and total protein radioactivity of the livers between the experimental and control animals. In experiment 460 a group of stock-fed rats, of weights comparable to those of the animals fed the 0.25% threonine-deficient diet for 3 weeks, received 2.5 μCi ^{14}C-leucine intraperitoneally 1 hour before killing. There was an increase in radioactivity of total protein in the liver and plasma and a decrease in muscle and spleen of the experimental group in comparison with the stock-fed control group.

Table 4 presents data of ^{14}C-leucine incorporation after a 5 minute pulse into organ proteins of rats force-fed the complete or 0.25% threonine-deficient diet for 4 weeks. After this short pulse hepatic protein incorporation was increased and muscle protein was decreased in experimental animals in comparison with controls. In this portion of the experiment, in addition to measuring liver acid-soluble radioactivity, free amino acids and specific activity of leucine in the livers were measured. The radioactivity of the precursor free amino acid in the livers at killing (5 minutes after injection) was calculated and the total liver protein radioactivities were corrected for these values. The percentage increase for corrected liver protein radioactivity in the experimental animals over that in control animals was 28%. This percentage increase was similar to that obtained after correction for total acid-soluble radioactivity, which was 30%.

In experiment 383 one group of animals which had been force-fed the 0.5% threonine-deficient diet for 28 days was then switched to a threonine-devoid diet for 4 days. In experiment 460 one group of animals which had been force-fed the complete diet for 25 days was then switched to a threonine-devoid diet for 3 days. The results for these groups (tables 1, 2 and 3) indicate that these animals responded to a threonine-devoid diet as did rats in earlier studies (1, 6–8).

In experiments 383 and 460 the liver, pancreas, submaxillary gland, parotid, stomach, adrenal, heart, kidney, thymus and lung were examined histologically in all animals. No pathologic changes were observed in the animals force-fed the complete diet. Experimental animals force-fed the 0.25% threonine-deficient diet showed enlargement of nucleoli of hepatic cells and atrophy of the pancreas, submaxillary gland, parotid, stomach and thymus. Oil red O staining of liver sections failed to reveal an increase in lipid in the majority

TABLE 4

Incorporation of ^{14}C-leucine 5 minutes after intraperitoneal administration into protein of liver, spleen, kidney and gastrocnemius muscle of rats force-fed complete or threonine-deficient diet for 28 days

Group [1]	No. of rats	Liver protein		Spleen protein [2]		Kidney protein [2]		Gastrocnemius muscle protein [2]	
		S.A.[3]	*cpm/liver/100 g body wt × 10^{-3}*	S.A.[3]	*cpm/spleen/100 g body wt × 10^{-3}*	S.A.[3]	*cpm/kidney/100 g body wt × 10^{-3}*	S.A.[3]	*cpm/muscle/100 g body wt × 10^{-3}*
C	2	229 ± 39 [4]	224 ± 14 [4]	139	8.09	119	11.7	37.6	5.74
TD ¼	2	457 ± 68	527 ± 70	179	8.06	133	14.8	26.3	2.98
% difference		+100	+135	+29	−0.4	+12	+27	−30	−48

[1] C indicates complete diet; TD ¼ indicates threonine-deficient diet containing 0.25% threonine. All animals received 3 μCi ^{14}C-leucine intraperitoneally.
[2] Values based on determinations of pooled specimens.
[3] S.A. = specific activity (cpm/mg protein).
[4] Mean ± SE of mean.

of animals. A few animals had a minimal amount of stainable deposits of lipid. Similar but less marked changes were observed in animals force-fed the 0.5% threonine-deficient diet. The changes were present after 2 weeks but were somewhat more marked after 4 weeks. Two groups of animals which were changed to a threonine-devoid diet for 3 or 4 days after having been fed the complete or 0.5% threonine-deficient diet for 25 to 27 days revealed a periportal fatty liver with enlargement of hepatic nucleoli and atrophy of the pancreas, submaxillary gland, parotid, stomach and thymus. These changes which were more advanced than in the other experimental groups were similar to those previously described (1, 6, 8).

DISCUSSION

In earlier studies (1–3) we investigated the pathologic changes in young rats induced by force-feeding a purified diet devoid of a single essential amino acid for 1 week. The present study was undertaken to observe the relative importance of complete versus partial absence of threonine in inducing a nutritional imbalance leading to the appearance of a kwashiorkorlike condition.

The previous studies demonstrated the importance of various factors such as the quantity of diet intake (1, 2, 7), the quantity of carbohydrate intake (10), the presence of lipid in the diet (11) and adrenalectomy (6) or hypophysectomy (12) on the induction and severity of pathologic changes. Dietary amino acids were also shown to be important, since rats force-fed an amino acid-free diet for 3 days showed no pathologic changes in the liver (13). In contrast, rats force-fed a threonine-devoid diet containing 9, 16 or 28% amino acids all developed similar pathologic changes (8). Animals force-fed a threonine-deficient diet for periods up to 4 weeks do not develop the same pathologic changes in the liver as were found in rats that are force-fed a threonine-devoid diet for 3 to 7 days. Thus, it is apparent that the absolute quantity of threonine in an otherwise balanced diet may play an important role in the induction of certain pathologic changes such as fatty liver. The

complete absence of threonine induces a fatty liver (1, 6, 8), whereas low amounts such as 0.25% DL-threonine are either completely or partially protective, under our experimental conditions.

In addition to the failure to observe a fatty liver in animals force-fed a threonine-deficient diet, the experimental animals in our present study did not demonstrate a significant elevation in hepatic glycogen in experiments of 1 to 4 weeks in duration, unlike animals force-fed a threonine-devoid diet for 3 to 7 days (1, 6, 7). Failure to observe increases in hepatic glycogen in two groups of animals that were shifted to a threonine-devoid diet, after previously having been fed the complete or 0.5% threonine-deficient diet (table 2), is difficult to explain, but may possibly be related to a lengthy preconditioning to the purified diet. These dissimilarities suggest that the degree of dietary threonine deficiency is critical in inducing alterations in lipid and carbohydrate metabolism in the liver.

The results of this study reveal some other differences, as well as some similarities, in the changes induced in animals force-fed a threonine-deficient diet for up to 4 weeks and those force-fed a threonine-devoid diet for up to 1 week. Rats force-fed the threonine-devoid diet have a high mortality within a week (1), while those force-fed the threonine-deficient diets survive well for periods up to 31 days. Also, the threonine-devoid group of animals gained less weight (1) than did animals fed similar quantities of the threonine-deficient diets. The enhanced hepatic protein synthesis in animals force-fed a threonine-deficient diet (table 3) is similar to that reported earlier with animals force-fed a threonine-devoid diet (5–8). This suggests that even relatively minor alterations in dietary threonine intake can influence hepatic protein synthesis. In other nutritional-deficiency studies, increased hepatic protein synthesis has been reported in rats fed diets deficient or devoid of proteins or amino acids (14–18). This change in the liver may be accompanied by decreased protein synthesis in other organs, particularly skeletal muscle (3, 8, 15, 16, 19). The ability of the liver to respond rapidly with enhanced protein synthesis has been demonstrated in a number of studies where fasted animals were given a single feeding of protein, complete amino acids or tryptophan (20–25).

A comparison of the findings between animals force-fed a threonine-devoid diet and those force-fed a threonine-deficient diet in our laboratory raises several important points which must be considered before one can compare data from different laboratories using various types of "threonine-deficient" diets. The composition of the diets used, the mode of feeding (ad libitum or force-feeding) and the duration of the experiments, as well as the amount of threonine in the diet, the amounts of the other amino acids and the quantity of diet consumed, are important variables. Caution must be exercised before attaching meaningful interpretations to vague terms such as "threonine-deficiency," since numerous variables can play important and influential roles in the experimental results. Altered states induced by various forms of nutritional imbalances should not be oversimplified by simple terms implying simple or single cause and effect relationships.

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Vitamin A Requirement of the Guinea Pig

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ABSTRACT This study was undertaken to establish the dietary level of vitamin A best adapted to the growth and maintenance of the young guinea pig. Growth records were kept and plasma and livers were analyzed for vitamin A. Histological examinations of tissues of the eye, trachea, kidney and liver were made. Optimal growth occurred when the level of vitamin A was between 1.67 and 9.9 mg/kg of diet. Significant storage of the vitamin in the liver began at a level between 6 and 7 mg/kg of diet, and the quantity stored increased rapidly with further increase in the level of vitamin A in the diet. No such pattern was found in the levels of vitamin A in the plasma. Levels of 1.67 and 3.3 mg of vitamin A/kg, although maintaining normal growth, did not prevent metaplasia of epithelial tissue in all animals.

This study was undertaken to establish the vitamin A requirement of the growing guinea pig and also, to describe the results of a dietary insufficiency and excess of this vitamin. Committee reports of the National Research Council (1) and the Canadian Department of Agriculture (2) indicate little new work on this subject since that of Bentley and Morgan (3) in 1945, which showed that a daily intake of 0.2 mg of vitamin A was the minimum level to allow its detection in the liver. The control diet formulated by Reid and Briggs (4) contains 6.0 mg/kg which means, if we assume a daily food consumption of about 30 g, an intake of 0.18 mg/day. From all indications, this level appears satisfactory for the guinea pig for growth and optimal health.

Recently, Howell et al. (5) studied changes in tissues of guinea pigs on diets containing methyl retinoate, with one set of controls on a diet free from vitamin A or its derivatives, and another set to which retinyl acetate (0.5 mg/animal) was administered in oil by pipet twice weekly. In view of their detailed report a relatively brief note indicating our different objectives and conclusions will suffice here. We are in general agreement where comparisons are relevant.

Our first experiment was designed to answer four main questions: 1) The time needed to deplete young animals of the vitamin A reserve that might have been built up prior to weaning; 2) to look for symptoms useful in assessing progressive stages of vitamin A deficiency; 3) to determine the relative efficiencies of different levels of vitamin A (retinol) to restore growth; and 4) to determine whether a relationship exists between liver and plasma levels and dietary intake.

In a second experiment, several groups of guinea pigs were compared for their ability to thrive on graded intakes of the vitamin, with doses ranging from zero to toxic levels in the diets.

EXPERIMENTAL

Animals. Male short-hair albino guinea pigs of the Hartley strain, were received from Fort Detrick, Maryland. They were reared individually in wire-bottomed cages and given food and water ad libitum. To avoid weight losses, they were fed a commercial ration [3] the first day, with increasing proportions of the experimental diet mixed with the ground ration in succeeding days. In this way, they adjusted to the diet change in the course of a week without setbacks of any consequence. It meant however that whereas Howell et al. (5) began with weanling guinea pigs with an average weight of about 200 g, ours were appreciably bigger before the experiment could be started. This undoubtedly is responsible for certain differences which exist between the two sets of findings. Ours

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began with a total liver storage of about 90 μg of vitamin A (average for 3 animals) with individual levels of 8.4, 7.2 and 7.6 μg of vitamin A/g liver. Under natural conditions, it appears that liver storage in the guinea pig may be low, about 3 μg/g of moist liver (see 6).

Diets. The vitamin A-free diet was a modification of the diet of Reid and Briggs (4). It contained, in grams per kilograms of diet: vitamin-free casein, 300; powdered sucrose, 246; glucose-hydrate, 170; cellulose (powdered cellophane), 150; mineral mixture (7), 75; corn oil, 50 (containing the fat-soluble vitamins); L-arginine·HCl, 3; ascorbic acid, 2; choline chloride, 2; and inositol, 2. In addition, fat-soluble (in corn oil) and B vitamins (in glucose-hydrate) were added at the levels used by Liu et al. (7), with 16 mg thiamine·HCl/kg present. The corn oil used was analyzed for carotene and none was found. Crystalline retinyl palmitate in corn oil was used as the source of vitamin A in these experiments.

Another group of animals received the commercial ration [3] as an additional control.

Vitamin A assays. The quantity of vitamin A in the diet was estimated from the stated potency of the oil and checked by spectrophotometry. In liver and plasma it was estimated by use of trifluoroacetic acid in chloroform at 620 mμ, essentially by the method of Neeld and Pearson (8). Because of very low levels in the plasma, certain modifications were necessary. As much blood as possible was obtained by heart puncture before autopsy. After centrifugation, the serum was mixed with ethanol and exhaustively extracted with petroleum ether. This could then be concentrated to a small volume, and an aliquot taken for the determination.

RESULTS

Growth experiments. In the first experiment there were 20 animals. Three were killed at the start, 10 were placed on the vitamin A-free diet, and 7 received a diet containing 6 mg vitamin A/kg diet. The initial weights averaged 360 g. Until day 27 there was no noticeable difference in growth rates, after which those on the vitamin A-free diet lagged behind, and after day 41 weight losses became more apparent. Thus, on day 46 the average had fallen from 595 to 565 g, whereas those receiving vitamin A continued to gain, weighing an average of 650 g on day 46. Growth was resumed by animals after 40 days on the vitamin A-free diet when placed on diets containing 3.3 to 33 mg of the vitamin/kg diet, but at levels of 66 mg/kg and above there was loss of weight.

In animals kept on the deficient diet for 7 to 8 weeks, few external symptoms of deficiency were discerned. The hair was slightly more unkempt, and some developed scales on the ears and on the dorsal surface of the feet. So far as could be detected, vision was unimpaired and this was confirmed by subsequent histological examination of the retinas.

In the second experiment, groups of 5 animals each were placed on diets containing 1.67, 3.3, 6.6, 9.9, 16.5, 165 and 330 mg retinol/kg diet. Starting weights averaged 258 g. Growth ceased after 21 days at zero intake and remained unchanged for the next 18 days, after which losses in weight were noted. Growth averages for all animals in each group are given in footnote 2, table 1. At dietary levels of 1.67 and 3.3 mg/kg the range of averages was from 516 to 537 g with no clear-cut evidence to favor one intake over another. At zero and toxic (330 mg vitamin A/kg diet) levels, the averages were 364 and 425 g, respectively.

Plasma levels. The plasma from 23 animals was analyzed for vitamin A. Levels of 0.05 to 0.10 μg of vitamin A/ml plasma were found in 4 out of 15 fed diets containing from zero to 6.6 mg vitamin A/kg diet. In 6 out of 8 animals on diets containing 9.9 mg/kg diet or above, the plasma levels were 0.08 to 0.4 μg/ml.

Liver storage. It appears worthwhile to show the individual analyses (table 1) to indicate not only the variation within a group, but to show also that storage of vitamin A in the liver becomes significant only when the vitamin concentration is 6.6 mg/kg or higher. In fact, at the optimal level for growth, it is not evident that significant storage has occurred.

Hematology. Normal red blood cell counts, hematocrit and hemoglobin were

TABLE 1

Vitamin A content of individual guinea pig livers, as affected by intake

Diet content	Approximate daily intake	Days on diet	Vitamin A content	
mg/kg	*mg*		*μg/g* liver	*μg* total
After adapting to diet		0 [1]	8.4 [2]	89.3
			7.2	82.0
			7.6	94.0
0		40	0 [3]	—
1.67	0.05	40	0.18	4.8
		40	0.10	2.1
		49	0.20	5.1
		49	0.30	6.7
		56	0.20	4.0
3.3	0.10	40	0.71	9.8
		40	0.26	4.7
		49	0.55	13.1
		49	0.57	17.0
		56	1.4	25.2
6.6	0.20	40	5.6	147.0
		40	1.4	32.5
		49	2.5	65.0
		56	17.0	289.0
9.9	0.30	40	22.1	590
		40	26.0	592
		49	33.5	880
		49	20.0	544
		56	9.0	238
330.0	10.0	40	2,000.0	26,000
		40	2,060.0	35,000
		56	1,094.0	19,700
		56	1,590.0	28,600
		60	2,030.0	39,000
(—) [4]	Commercial ration	40	12.1	242 [5]
		40	6.4	128
		40	8.0	160
		56	15.0	300
		56	9.0	180

[1] Seven days were allowed after receipt of the animals for adaptation to the diet.

[2] Values for individual animals are listed to show the degree of variability. Growth averages at day 40 were: 364 ± 23.9, 516 ± 17, 537 ± 30.4, 527 ± 18.8, 425 ± 24.3, and 555 ± 12.8 for dietary levels of zero, 1.67, 3.3, 6.6, 9.9 and 330 mg of vitamin A/kg, and for the commercial ration (Purina Chow).

[3] For all 5 animals.

[4] Contained 12 IU/g vitamin A (presumably as carotene in the alfalfa).

[5] An arbitrary figure was set here for liver weight. The actual weights were apparently not recorded for this group.

found in the deficient animals. Though the diet is adequate in all respects except for vitamin A, it is possibly not surprising that no abnormality should be observed in this respect.

Histology. Tissues to be examined were excised and samples were fixed in 5% formalin. These included: a small ring of trachea at the bifurcation point; a gram of liver from the lower edge of a lobe; the left kidney, sliced through the midlongitudinal plane to cut open the calyx end of the ureter; the left eye, exorbitated by excision of the optic nerve, and tearing of the external muscles, and fixed as such.

Slide mounts were made from paraffin and from frozen sections, and stained.[4] Findings may be summarized as follows:

Eye. The scleral and corneal mucous membranes, known to become keratinized

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during vitamin A deficiency in rats still appeared normal.

Tracheal epithelium revealed squamous metaplasia, severe in the deficient animals, and detectable at dietary levels of 1.67 and even 3.3 mg vitamin A/kg diet.

Kidney. Metastatic calcinosis was clearly evident at the toxic level only. Hyperplastic epithelium of the collecting tubules and degeneration were evident in deficient animals, and in one animal at the toxic level. The pelvic mucous membrane was replaced by a flattened nonfunctional thin layer of cells in deficient animals and signs of derangement could be detected when the diets contained low and toxic levels of the vitamin, namely 3.3 and 330 mg, respectively, per kilogram diet.

Liver. Atrophy, as revealed by reduced cytoplasmic content, and increased mitosis usually appear together. The atrophy was severe at zero and toxic levels, and was detected in one animal on the diet containing 3.3 mg of vitamin A/kg. Metastatic calcinosis was found only at the toxic level. Fat infiltration was strongly marked in deficient animals, and was detected also at the toxic level.

DISCUSSION

These tests show that levels of vitamin A ranging from 1.67 to 9.9 mg/kg diet maintained normal growth. There was no advantage, in terms of growth response, to levels above 6.6 mg/kg, although they resulted in a marked increase in storage of vitamin A in the liver. Significant growth impairment of animals occurred at deficient and toxic levels, and this is a manifestation of pathological disturbance.

In the deficient animals, there was marked metaplasia of epithelial cells, atrophy of liver cells and fatty degeneration of the liver. Even dietary levels of 1.67 and 3.3 mg vitamin A/kg did not invariably suffice to preserve fully the integrity of tissues such as secretory epithelia and liver cells.

It may be concluded that a diet containing between 6 and 7 mg of vitamin A/kg meets the requirement of the growing guinea pig for this vitamin. On the basis of 30 g of food consumed daily, this is equivalent to 0.2 mg of vitamin A, and is in agreement with the recommendations of Reid and Briggs (4).

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Amylase, Procarboxypeptidase and Chymotrypsinogen in Pancreas of Chicks Fed Raw or Heated Soybean Diet

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ABSTRACT Levels of amylase, procarboxypeptidase and chymotrypsinogen were measured in the pancreas of chicks fed diets containing heated or raw soybeans for several days after hatching. In chicks continuously fed raw soybeans, the levels of all three enzymes were lower by 50% or more than those in control chicks fed heated soybeans. This difference was apparent after only 1 day of feeding. In chicks fasted for 1 day after feeding raw soybeans for 4 days, levels of chymotrypsinogen and procarboxypeptidase were about twice those in control chicks, whereas amylase levels were the same as those of the controls. It is suggested that raw soybeans may have two different effects on the pancreas which are not directly related: 1) they increase the secretion of digestive enzymes in general, and 2) they specifically increase the synthesis of proteolytic enzymes.

Raw soybeans support the growth of animals more poorly than heated soybeans and cause enlargement of the pancreas. Extensive research has been carried out to identify the agent(s) in raw soybeans causing these effects (1). There is strong evidence that pancreatic enlargement is caused by the trypsin and chymotrypsin inhibitors present in raw soybeans (2–6). Some experiments indicate, however, that there may be factors other than inhibitors of proteolytic enzymes in raw soya which can give rise to pancreatic enlargement (7).

The nature of the effect(s) of raw soybeans on the pancreas is still not clear. Some authors have claimed that pancreatic enlargement results from hyperplasia (8) while others have presented evidence that there is hypertrophy (9, 10). Feeding of raw soybeans increases the secretion of pancreatic digestive enzymes to rates greatly above normal. This has been concluded from increased levels of digestive enzymes in the intestine, and decreased steady-state levels of enzymes in the pancreas (11–16). When animals are fasted after feeding raw soybeans, however, levels of proteolytic enzymes in the pancreas are elevated above levels in control animals fed heated soybeans (16–18). Previous work has shown that whereas the fasting levels of proteolytic enzymes increased after feeding raw soybeans the levels of amylase were unaffected or fell (15, 16, 18). In most of these investigations amylase and proteolytic enzyme activity were each measured in a different series of experiments. The aim of the present work was to determine in the same series of experiments how the levels of three digestive enzymes (amylase, chymotrypsin(ogen) and (pro)carboxypeptidase) were affected by continuous feeding of raw soybeans or by feeding raw soybeans followed by fasting. It was shown that continuous feeding of raw soybeans decreases the steady-state levels of all three enzymes below the control levels to about the same degree. In animals fasted after feeding raw soybeans, however, only the levels of the proteolytic enzymes but not that of amylase are elevated above the levels in controls fed heated soybeans. These observations suggest that raw soybeans have a dual effect on the pancreas.

MATERIALS AND METHODS

Soybean meal. Soybean flakes defatted at 40 to 55° with light petroleum (boiling range 40 to 65°) were the gift of Shemen Israel Oil Industries Ltd. Heated or un-

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heated flakes were pulverized with a Waring Blendor.

Heating of soybean flakes. Soybean flakes, spread on flat trays to a depth of 4 cm or less, were heated for 40 minutes at 120° in an autoclave. This treatment yielded soybean meal which gave maximal adsorption of cresol red (19). Chicks fed soybean meal heated in this manner grew at rates similar to chicks on a commercial diet and showed no pancreatic enlargement.

TABLE 1

Composition of diets

	%
Soybean flour (raw or heated) [1]	50.0
Cornflour	33.4
Safflower oil	10.0
$CaCO_3$ [2]	1.0
$Ca_3(PO_4)_2$ [2]	2.0
NaCl	0.5
DL-Methionine	0.6
Vitamin mixture [3]	2.5

[1] For details of preparation see Materials and Methods.
[2] Technical.
[3] Produced by Hamashbir, Israel. Contained: (per g) vitamin A, 3,000 IU; vitamin D, 400 IU; vitamin B_{12}, 4 μg; riboflavin, 1.6 mg; calcium pantothenate, 3.2 mg; vitamin K, 0.8 mg; vitamin E, 0.4 IU; choline chloride, 60 mg; niacin, 8 mg; and antioxidant BHT, 50 mg.

Composition of the diets. Table 1 summarizes the composition of the raw and heated soybean diets.

Chicks. White Leghorn × New Hampshire chicks were kept in electrically heated brooders. Water was given ad libitum at all times.

Preparation of homogenates. Pancreases were homogenized either in 0.03 M phosphate buffer pH 6.9, or in 0.01 M tris-HCl buffer pH 7.6 to give a protein concentration of about 10 mg/ml. Before activation of proteolytic enzymes homogenates were diluted with 0.01 M tris-HCl buffer pH 7.6 to give a protein concentration of 1 mg/ml. Homogenates were stored for not more than 48 hours at 0° or for longer periods at −20°. Frozen homogenates were never thawed more than once. Under these conditions all three enzymes were stable.

Analytical methods. Protein was determined by the method of Lowry et al. (20) using crystalline bovine plasma albumin as standard. Amylase was determined according to Bernfeld (21), a unit being defined as the amount that in 3 minutes at 30° catalyzes the appearance of reducing groups equivalent to 1 mg of maltose hydrate. Chymotrypsinogen and procarboxypeptidase were activated with trypsin, and activities of the resulting chymotrypsin and carboxypeptidase were determined as described previously (22).

Enzymes and substrates. Bovine trypsin twice crystallized, salt-free and lyophilized was obtained from Worthington Biochemical Corporation, Freehold, New Jersey; *N*-acetyl-L-tyrosine ethyl ester was purchased from Yeda Co. Ltd., Rehovoth, Israel, and hippuryl-DL-phenyllactic acid was the product of Cyclo Chemical Corporation, Los Angeles, California.

RESULTS

Effect of raw soybeans on total pancreatic protein. Total pancreatic protein of chicks fed unheated soybeans was only slightly higher (10 to 20%) than that of control chicks fed heated soybeans. Because of inhibition of growth, however, ratios of total pancreatic protein to body weight were about 40% higher in chicks fed raw soybeans than in the controls.

Effect of continuous feeding of raw soybeans. Both total and specific activities of all three enzymes studied were considerably lower in chicks fed the raw soybean diet than in chicks fed the heated soybean diet (table 2). A clear-cut difference was observed after only 1 day of feeding. The ratios of enzyme levels in pancreas of chicks fed raw soybeans to those in pancreas of chicks fed heated soybeans were lower (about 1:3) after 1 day of feeding than after 4 days of feeding (about 1:2). At each feeding time, levels of all three enzymes were decreased below the control levels to about the same degree.

Effect of feeding raw soybeans followed by fasting. After feeding for 4 days and fasting for 1 day specific activities of carboxypeptidase and chymotrypsin were about twice as high in homogenates of pancreas from chicks fed the raw soybean diet as in controls fed the heated soybean diet (table 3). Specific activities of amylase, however, were not significantly different in pancreas of chicks on the two diets. Differences in proteolytic zymogen levels between chicks fed raw soybeans and controls

TABLE 2

Activities of amylase, carboxypeptidase and chymotrypsin in homogenates of pancreas from chicks continuously fed raw or heated soybean diet [1]

Time on diet	Enzyme	Diet			
		Heated		Raw	
		Total activity [2]	Specific activity [2]	Total activity [2]	Specific activity [2]
days		*units/ pancreas*	*units/mg protein*	*units/ pancreas*	*units/mg protein*
1 [3]	Amylase	970 ± 270	61 ± 14	260 ± 130	13 ± 6
	Carboxypeptidase	91 ± 30	5.6 ± 1.4	30 ± 15	2.0 ± 1.3
	Chymotrypsin	290 ± 75	17.4 ± 3.8	87 ± 17	5.4 ± 1.9
4 [3]	Amylase	1870 ± 390	65 ± 15	950 ± 185	31 ± 10
	Carboxypeptidase	128 ± 37	4.4 ± 1.6	67 ± 14	2.1 ± 0.7
	Chymotrypsin	350 ± 120	11.5 ± 1.2	200 ± 43	6.3 ± 1.6

[1] Chicks fasted for 1 day after hatching were fed ad libitum for the period shown. Pancreases from 5 chicks of each group (namely, same diet and same time of feeding) were pooled and homogenized. Activities of amylase, carboxypeptidase and chymotrypsin were determined in the same homogenates in each case.
[2] Mean ± SD.
[3] Values for both periods of feeding are the mean of 4 experiments.

TABLE 3

Activities of amylase, carboxypeptidase and chymotrypsin in homogenates of pancreas from chicks fasted after feeding raw or heated soybean diet [1]

Enzyme	Diet			
	Heated [2]		Raw [3]	
	Total activity [4]	Specific activity [4]	Total activity [4]	Specific activity [4]
	units/ pancreas	*units/mg protein*	*units/ pancreas*	*units/mg protein*
Amylase [5]	2290 ± 690	105 ± 19	2810 ± 1150	105 ± 16
Carboxypeptidase [6]	119 ± 40	5.8 ± 0.9	262 ± 43	9.9 ± 1.0
Chymotrypsin [6]	224 ± 70	8.9 ± 4.1	472 ± 114	18.1 ± 1.7

[1] Chicks fasted for 1 day after hatching were fed for 4 days after which they were fasted for 26 hours before they were killed and the pancreas removed. Pancreases from 5 chicks of each group were pooled and homogenized in each experiment.
[2] Total protein per pancreas = 22.0 ± 6.7 mg.
[3] Total protein per pancreas = 24.0 ± 4.4 mg.
[4] Mean ± SD.
[5] Mean values of 5 experiments for heated and 6 experiments for raw soybean diet.
[6] Mean values of 4 experiments for heated and 5 experiments for raw soybean diet.

reached a maximum only after 3 days of feeding followed by 1 day of fasting.

DISCUSSION

Lepkovsky et al. (15, 16) have shown that continuous feeding of raw soybeans to chicks lowers the levels of amylase and proteolytic enzymes in the pancreas below those found in chicks fed heated soybeans. These workers were unable, however, to determine whether the levels of amylase were depressed to the same degree as those of proteolytic enzymes, since levels of the two types of enzyme were measured in a different series of experiments. In the present paper specific activities of three enzymes, amylase, carboxypeptidase and chymotrypsin, were measured in the same series of experiments and it was shown that continuous feeding of raw soybeans decreased levels of all three enzymes to about the same degree below the control levels (table 2). This observation was in contrast to the finding that in pancreas of chicks fasted after feeding raw soybeans specific activities of proteolytic enzymes, but not that of amylase, were elevated above control levels (table 3). Our results, using specific methods for two types of proteolytic enzymes, confirm the observa-

tions of other investigators who showed that in chicks fasted after feeding raw soybeans proteolytic enzyme (zymogen) levels are elevated above control levels whereas the level of amylase is the same as, or less than, that in the controls (15–18). The data in this paper confirm the finding of Lepkovsky et al. (15) that when the diet was supplemented with methionine, pancreatic amylase levels were the same in chicks fasted after feeding heated or raw soya.

It seems paradoxical that, although continuous feeding of raw soybean diet caused depletion of all digestive enzymes studied, only the levels of the proteolytic enzymes were elevated above those of the control after fasting. Reduction of pancreatic enzyme levels below normal by raw soybean diet has been ascribed to the accelerated secretion of digestive enzymes (11–16). If increased stimulation of secretion leads to increased fasting levels of digestive enzymes, one would expect the levels of all the enzymes studied, including amylase, to increase in the same manner. It seems unlikely, however, that a general stimulation of secretion of amylase, procarboxypeptidase and chymotrypsinogen would lead to a selective elevation of the fasting levels of only the latter two enzymes without affecting amylase. A more attractive hypothesis is that raw soybeans have two different effects on the pancreas which are not directly related: 1) They increase secretion of digestive enzymes in general, and 2) they specifically increase the synthesis of proteolytic enzymes.

While differences in the fasting levels of proteolytic enzymes between chicks fed raw and heated soybeans reach a maximum after several days of feeding, a marked lowering of digestive enzyme levels, presumably due to accelerated secretion, may be observed after only 5 hours of feeding raw soybeans (16). Perhaps the stimulation of secretion by raw soybeans represents a short-term response to compensate for lowered proteolytic activity in the gut (5, 23), whereas the selective induction of proteolytic enzyme synthesis represents a long-term adaptation to the trypsin inhibitors in the diet. Both responses, short-term and long-term, would serve to raise the proteolytic activity in the gut. The long-term effect would also help to lighten the secretory load on the pancreas since, if the pancreatic juice is enriched with proteolytic enzymes, less would have to be secreted to compensate for trypsin inhibitors in the diet. It is possible, however, that the two different effects of raw soybeans are not both ascribable to trypsin inhibitors but are due to entirely different factors. Such a hypothesis would explain reports that soybean fractions free of trypsin inhibitors can cause pancreatic enlargement (7). Experiments to test the effects of pure trypsin inhibitors on pancreatic enzyme levels after continuous feeding or after feeding followed by fasting should help to answer this question.

Although the specific activities of amylase were the same in chicks fed raw soybeans as in controls, amylase-to-body weight ratios were considerably higher in the former group (cf. also Lepkovsky et al. (15)). This means that the enlargement of the pancreas involves an increase in the absolute amounts of digestive enzymes in general, in addition to the selective increase in amounts of proteolytic enzymes. Thus, the specific effect of raw soybeans on the synthesis of proteolytic enzymes seems to be superimposed on a more general increase of total pancreatic protein (including amylase). It is tempting to suggest that the nonspecific increase of pancreatic protein is related to the generalized stimulation of digestive enzyme secretion by raw soybeans, whereas the specific effect on proteolytic enzymes is the result of some more specific homeostatic mechanism controlling proteolytic activity in the intestine. A regulatory mechanism adjusting pancreatic function to proteolytic activity in the gut has been proposed previously by other investigators (3, 17, 23). It seems plausible that the lowering of proteolytic activity in the intestine by soybean trypsin inhibitors may have a selective-inductive effect on the synthesis of proteolytic enzymes in the pancreas. Synthesis of proteolytic enzymes by the pancreas has been shown before to be selectively stimulated by enrichment of the diet with protein (24).

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Effect of Diet, Dietary Regimens and Strain Differences on Some Enzyme Activities in Rat Tissues

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ABSTRACT The activities of aldolase, glucose 6-phosphatase, glucose 6-phosphate dehydrogenase, alkaline phosphatase and β-glucuronidase were measured in tissues of two strains of rats (Wistar and BHE) fed a stock diet or a diet containing 25% of whole egg (SPE), to assess the influence of diet and heredity on metabolism. Feeding the SPE diet resulted in increased liver and perirenal fat pad weights and liver protein in both strains of rats. Liver glucose 6-phosphate dehydrogenase activity was higher in all groups fed the stock diet whereas glucose 6-phosphatase and aldolase activities were higher in all groups fed the SPE diet. The BHE rats had higher liver glucose 6-phosphate dehydrogenase and β-glucuronidase activities but lower liver and serum alkaline phosphatase activities than Wistar rats receiving similar treatment. Fasting for 16 hours resulted in lower liver aldolase and liver, serum and kidney alkaline phosphatase activities in both strains. Only Wistar rats fed the SPE diet respired the carbon-1 on glucose more rapidly than the carbon-6. The Wistar rats fed the SPE diet had the lowest activity of glucose 6-phosphate dehydrogenase in the liver; they apparently used the hexose monophosphate shunt more actively than the other groups. The total respired activities from glucose-U-^{14}C were similar in each group.

Previous investigations from this laboratory showed that diet may accelerate the development of kidney damage, fatty infiltration of the liver, lipemia and elevated levels of serum cholesterol in a strain of rats (BHE) used in this laboratory (1, 2). A diet containing 25% cooked dried egg (SPE) was particularly effective in this respect. The age at which these changes occurred in the BHE rats fed the egg diet depended on the type of carbohydrate in the diet (3). In contrast, the Wistar rat seldom developed kidney damage, regardless of the diet (1). Further evidence suggested that the fat metabolism of this strain of rat differed from that of the BHE rat (4).

The cause of the differences in the response to diet of these two strains is unknown. Control of metabolic processes has often been found to reside in the activity of one or more key enzymes in a metabolic pathway; therefore, the activities of a number of enzymes have been measured in the tissues of BHE and Wistar rats, in an attempt to characterize and explain some of the differences in metabolic control of these two strains. The influences on enzyme activities of feeding a stock diet that has proved satisfactory for development and survival of our stock animals and the SPE diet which causes early death of BHE rats were compared. Enzyme activity in the tissues may be influenced by prior feeding experience or by the fluctuation of diurnal eating pattern. To learn if such effects were important, fasting and controlled patterns of feeding were studied for their influence on these enzyme activities. Since major differences in glucose 6-phosphate dehydrogenase (G-6-PD) activities were observed, studies of rates of oxidation of glucose labeled with ^{14}C in various positions were included as a measure of the relative importance of the hexose monophosphate pathway in the metabolism of these animals.

EXPERIMENTAL

Rats and diet. Male weanling rats of the BHE and Wistar strains were obtained from the same closed colony used in other studies from this laboratory (1–3). Experimental animals were taken only from litters that had six or more males. Six rats from each of ten litters were assigned ran-

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domly to each of 6 experimental treatments (10 rats in each treatment).

Rats were housed individually in wire cages in air-conditioned quarters maintained at approximately 25° and a relative humidity of 50%. Rats were fed ad libitum from weaning either a stock diet [1] or the SPE diet which contained: (in parts/100 g) casein, 12; lactalbumin,[2] 6; yeast,[3] 7.5; beef tallow, 6; salt mix, 3; whole egg powder, 25; sucrose, 39; and cellulose flour, 1.5. The analytical data of the SPE diet contained the following: (in grams/100 g) protein, 30; fat, 17; ash, 3; moisture, 5; and carbohydrate by difference, 45. The corresponding values for the stock diet [4] were protein, 24; fat, 7; ash, 7; moisture, 7; and carbohydrate by difference, 55. Rats fed the SPE diet received weekly supplements of 2 drops of percormorph oil supplying 395 IU of vitamin A and 56 IU of vitamin D daily, and 0.01 ml of cottonseed oil containing 36 mg *dl*-alpha-tocopheryl acetate, or 5.1 mg daily. Groups of 90-day old Wistar and BHE rats designated as "16-hour fasted," were deprived of all food but not water beginning at 4 PM on the day before killing. The animals were killed at 8 AM the following morning. Groups designated as "fasted and re-fed" were treated in the same manner as the fasted groups except that at 8 AM the food was returned to the cage and the rats were killed at 12 noon. A large amount (4 to 6 g) of diet was consumed during this 4-hour period of refeeding. Groups designated as "fed at intervals" had food removed at 4 PM when 86 days of age. At 8 AM, 12 noon, and 4 PM of the following three days these rats received one-third of their previous average daily intake. On day 4 they were fed in the same way, at 8 AM and 12 noon, but were killed at 3 PM. All rats were weighed before they were decapitated. Blood was collected. Liver, kidneys and epididymal and perirenal fat pads were quickly dissected and weighed. Organs and serum were kept in ice until homogenized for enzyme assay.

Chemical analyses. Portions of tissues were homogenized in a 1:10 (w/v) dilution, either with H_2O or with a pH 7.0 solution containing 150 mM KCl, 5 mM $MgCl_2$ and 6 mM EDTA, with a motor-driven Teflon pestle in a glass homogenizer for 1.25 minutes. Homogenates in water were used for all analyses except for glucose 6-phosphatase (G-6-Pase) and G-6-PD.

Enzyme determinations were carried out as soon as possible on the same day that the tissues were collected. Glucose 6-phosphatase was assayed by the method of Freedland and Harper (5). The activity of G-6-PD was measured by determining the rate of formation of TPNH (6) without the addition of 6-phosphogluconic acid to the reaction mixture. It is recognized that a small amount of the TPNH results from the reduction of 6-phosphogluconate. Löhr and Waller (7) suggested that TPNH oxidizing reaction (glutathione reductase and other reductases) tend to compensate for this. Alkaline phosphatase [5] and aldolase and β-glucuronidase (8) were measured by the color reactions of the final product. Tissue protein was analyzed by the method of Lowry et al. (9).

For studies of metabolism of labeled glucose or acetate,[6] animals were raised under the same conditions as used for enzyme-activity studies. At approximately 3 months of age (9 weeks on diet) animals were fasted overnight. At 8 AM the next day they were given 4 g of the diet they had been receiving ad libitum. One hour later each animal received intraperitoneally 2 mg of glucose-1-^{14}C containing 2 μCi dissolved in 0.5 ml isotonic saline. Immediately following the injection each animal was placed in an individual all-glass metabolic chamber and respired CO_2 collected in ethanolamine–ethylene glycol monoethyl ether (3:7). Six consecutive 1-hour collections of respiratory CO_2 were made. Rats were then returned to their cages and fed ad libitum for 56 hours. They were again fasted overnight, fed 4 g of diet and 1 hour later injected with 2 μCi of glucose-6-^{14}C (2 mg in 0.5 ml). Respired CO_2 was collected as before. After another 56 hours of feeding ad libitum the procedure was repeated a third time on the same animals

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[2] Obtained from The Borden Co.
[3] Dried brewer's yeast, type 200 B, from Standard Brands, Inc.
[4] Analyzed in our laboratory.
[5] The colorimetric determination of phosphatase. Sigma Chemical Company, St. Louis, Tech. Bull. no. 104, 1961.
[6] Nuclear-Chicago Corporation, Des Plaines, Illinois.

with 4 μCi of glucose-U-^{14}C (2 mg in 0.5 ml). In this case only four 1-hour collections of CO_2 were made. Another group of rats received an injection of 4 μCi of acetate-1-^{14}C (2.8 μg sodium acetate in 0.5 ml of isotonic saline). Radioactivity in collected CO_2 samples was measured by liquid scintillation counting using the mixture of Jeffay and Alvarez (10). Quenching was corrected by the use of an external standard.

RESULTS

The weights of total body, liver and fat pads, as well as total liver protein for both BHE and Wistar rats, are presented in table 1. From the analysis of variance it is clear that strain, diet and pattern of feeding had significant effects on liver weight and liver protein. Fasting for 16 hours in animals fed both diets resulted in lower body weights, liver weights and liver protein than for interval-fed rats. Refeeding for 4 hours did not eliminate these differences. Feeding of the SPE diet increased the liver weight, liver protein and weight of perirenal fat pads with each treatment over those of rats fed the stock diet. The effect of diet on body weight did not prove to be statistically significant. There were no significant differences in kidney weight (2.3 to 2.8 g) due to strain, diet or pattern of feeding at this age.

The activities of liver, kidney, serum enzymes and analyses of variance are presented in table 2. Since there was no interaction found between the variables, except liver G-6-PD between diet × strain ($P < 0.05$), the F values for interactions are not presented. The activities are reported for the total liver to compensate for the differences in liver weight produced by diet and pattern of feeding. The activity

TABLE 1

Body weights, and tissue weights and composition of two strains of rat fed stock or SPE diet

Feeding pattern	Diet	Body wt	Liver wt	Epididymal fat [1]	Perirenal fat [1]	Total liver protein
		g	*g*	*g*	*g*	*g*
			BHE			
Fasted	Stock	338 ± 14 [2]	10.1 ± 0.3	3.85 ± 0.6	4.76 ± 0.6	2.41 ± 0.10
	SPE	336 ± 21	13.0 ± 1.0	5.73 ± 1.7	7.58 ± 1.3	2.78 ± 0.20
4-hour refeeding	Stock	335 ± 18	10.7 ± 0.6	3.54 ± 0.4	3.97 ± 0.2	2.38 ± 0.15
	SPE	364 ± 14	15.5 ± 1.0	4.67 ± 0.6	6.34 ± 0.7	3.14 ± 0.17
Interval feedings	Stock	372 ± 16	12.1 ± 0.5	5.36 ± 1.1	5.77 ± 1.2	2.64 ± 0.12
	SPE	374 ± 9	17.0 ± 0.6	6.57 ± 0.6	7.32 ± 0.4	3.54 ± 0.15
			Wistar			
Fasted	Stock	332 ± 8	9.4 ± 0.4	5.16 ± 0.9	5.08 ± 1.1	2.28 ± 0.31
	SPE	322 ± 5	10.7 ± 0.2	4.83 ± 0.5	6.58 ± 0.8	2.49 ± 0.15
4-hour refeeding	Stock	338 ± 12	10.3 ± 0.5	4.92 ± 0.3	4.64 ± 1.1	2.29 ± 0.12
	SPE	317 ± 17	11.8 ± 0.8	4.22 ± 0.8	3.94 ± 0.7	2.41 ± 0.14
Interval feedings	Stock	356 ± 8	12.4 ± 0.4	4.49 ± 1.0	4.18 ± 0.4	2.69 ± 0.10
	SPE	353 ± 12	14.5 ± 0.7	4.85 ± 0.1	5.56 ± 0.6	3.00 ± 0.12
Analysis of variance F values						
Diet		0.01	83.11 **	1.61	9.80 **	29.94 **
Pattern of feeding		5.46 *	34.56 **	1.65	2.04	13.51 **
Strain		4.39 *	22.82 **	0.22	4.05 *	12.57 **
D [3] × S [4]		1.74	15.19 **	3.16	2.57	8.11 **
D × P [5]		0.13	18.24 **	0.51	0.65	1.46
S × P		0.18	0.72	1.54	0.67	0.58

[1] Average of 4 rats.
[2] Mean of 10 rats ± SE.
[3] D = diet.
[4] S = strain.
[5] P = pattern.
* Significant at P = 0.05 or less.
** Significant at P = 0.01 or less.

TABLE 2

Liver total enzyme activities and serum and kidney enzyme level in BHE and Wistar rats as influenced by diet and pattern of feeding

Feeding pattern	Diet	Glucose 6-phosphate dehydrogenase [1]	Glucose 6-phosphatase [1]	Aldolase [1]	Alkaline phosphatase [1]	β-Glucuronidase [1]	Serum alkaline phosphatase	Kidney alkaline phosphatase
							ml/hr	*g/min*
				BHE				
Fasted	Stock	40.8 ± 4.8 [2]	173 ± 55	80 ± 7	140 ± 14	1164 ± 130	5.4 ± 0.4	21.9 ± 1.7
	SPE	12.9 ± 0.9	181 ± 10	100 ± 14	121 ± 16	1107 ± 119	5.6 ± 0.6	16.8 ± 1.1
4-hour refeeding	Stock	36.8 ± 7.4	173 ± 8	69 ± 9	127 ± 27	937 ± 65	7.4 ± 0.6	21.5 ± 1.7
	SPE	21.5 ± 3.7	235 ± 13	137 ± 21	181 ± 28	950 ± 84	5.8 ± 0.6	19.0 ± 1.8
Interval feedings	Stock	46.4 ± 8.0	172 ± 7	84 ± 8	161 ± 10	1227 ± 96	9.6 ± 0.8	20.1 ± 1.5
	SPE	21.8 ± 2.7	234 ± 13	163 ± 21	200 ± 17	1131 ± 108	10.6 ± 1.0	17.8 ± 1.1
				Wistar				
Fasted	Stock	22.4 ± 0.8	162 ± 10	77 ± 9	266 ± 35	819 ± 49	7.2 ± 0.6	23.4 ± 2.3
	SPE	10.7 ± 0.6	183 ± 15	99 ± 14	232 ± 18	843 ± 75	8.8 ± 0.6	21.8 ± 1.8
4-hour refeeding	Stock	28.3 ± 5.0	164 ± 12	83 ± 8	278 ± 45	770 ± 39	13.0 ± 1.0	27.2 ± 3.1
	SPE	11.9 ± 1.0	170 ± 10	103 ± 13	210 ± 31	830 ± 68	11.4 ± 1.0	26.8 ± 3.4
Interval feedings	Stock	30.2 ± 2.2	194 ± 16	106 ± 13	304 ± 28	793 ± 52	23.4 ± 3.0	35.5 ± 6.9
	SPE	15.3 ± 0.6	244 ± 14	162 ± 17	351 ± 41	860 ± 50	26.2 ± 4.0	35.4 ± 7.4
Analysis of variance *F* values								
Diet		77.9 **	16.5 **	16.5 **	0.0	0.0	0.2	5.2 *
Feeding pattern		3.3	8.40 **	10.2 **	6.2 **	3.1	39.6 **	2.8
Strain		25.0 **	0.04	0.00	54.0	33.4 **	54.8 **	48.0 **

[1] Mean total liver enzyme activity, as μmoles product or TPNH produced per minute.
[2] Mean of 10 rats ± SE.
* Significant at $P = 0.05$ or less.
** Significant at $P = 0.01$ or less.

of kidney alkaline phosphatase per gram of tissue is presented since kidney weights were not altered by the experimental variables.

All groups fed the SPE diet had lower activity of liver G-6-PD, but had higher activities of liver G-6-Pase and adolase than those fed stock diet. The activities of liver G-6-Pase, aldolase, and alkaline phosphatase and serum alkaline phosphatase were generally lower in fasted rats than those on interval feeding. The 4-hour refeeding generally did not result in activities different from those in the fasted rats. Regardless of the diet and pattern of feeding, the activities of liver G-6-PD and β-glucuronidase of BHE rats were significantly higher than those of Wistar rats. In contrast, the activities of liver, serum and kidney alkaline phosphatase of Wistar rats were significantly higher than those of BHE rats. There was no apparent effect on kidney G-6-PD activity due to strain, diet or pattern of feedings.

The hourly recoveries of labeled ^{14}C in respired CO_2 following injection of labeled glucose are shown graphically in figure 1. Each animal received each labeled glucose, always administered in the same order (glucose-1-^{14}C, -6-^{14}C and -U-^{14}C). Since there was little difference in respired $^{14}CO_2$ after the first three collections between either diets or strains, the data of collections 4 to 6 are not presented.

There were no important differences in the recovery of ^{14}C from glucose-U-^{14}C as the result of strain or diet. The recovery from 6-carbon-labeled glucose, however, was much less than that from 1-carbon-labeled glucose, or uniformly labeled glucose, during the three hours following injection for the Wistar rats fed the SPE diet. The other groups showed a trend toward less recovery from 6-labeled glucose than from 1-labeled glucose, but was not of the magnitude found in the Wistar rats fed the SPE diet.

A similar experiment using different animals and acetate-1-^{14}C was also performed (not shown in tables). The recovery of respired $^{14}CO_2$ in 4 hours was 77% of the dose with each group.

DISCUSSION

The two diets used in this study differ in a number of aspects so it will not be possible to attribute any difference to a single dietary component. The importance of the study is to identify those enzyme systems and metabolic pathways which differ greatly in activity between the two diets and two strains of rats. These systems will then be studied using diets that vary only in single components.

Liver G-6-PD and β-glucuronidase activities were affected to only a small degree by patterns of feeding and fasting. This suggests that differences observed in the activity of these enzymes are not artifacts due to time of measurement but are characteristic of the metabolic patterns of the animal. The marked differences due to feeding patterns with liver aldolase, G-6-Pase and serum alkaline phosphatase show that the recent feeding experience of the rat has important effects on some enzyme systems.

The increased liver fat of BHE rats consuming the SPE diet reported by others (1, 2) paralleled the greater weights of fat pads and livers found here. Glucose 6-phosphate dehydrogenase is an important source in the cells of reduced TPN which is required for many synthetic processes, especially fat synthesis. The activity of this enzyme is known to adapt greatly to conditions that modify fat synthesis (11, 12). Increased fatty acid synthesis is generally associated with increased activities of this enzyme. Dietary sucrose has been reported to increase the activity of this enzyme in liver (13, 14). Fat, when present at levels of 15% or more, has been reported to reduce the activity of this enzyme (14). The SPE diet in this study contained the greater level of fat and consistently resulted in the lower activity of G-6-PD. The assumption must not be made that the activity of this enzyme parallels that of fatty acid synthesis in liver (11).

The tracer studies included in this report suggest a different picture. In the whole animal, a comparison of the rate of oxidation of carbon-1 of glucose with that of carbon-6 is often taken as a qualitative index of the direct oxidation of glucose 6-phosphate by G-6-PD. If this is a valid assump-

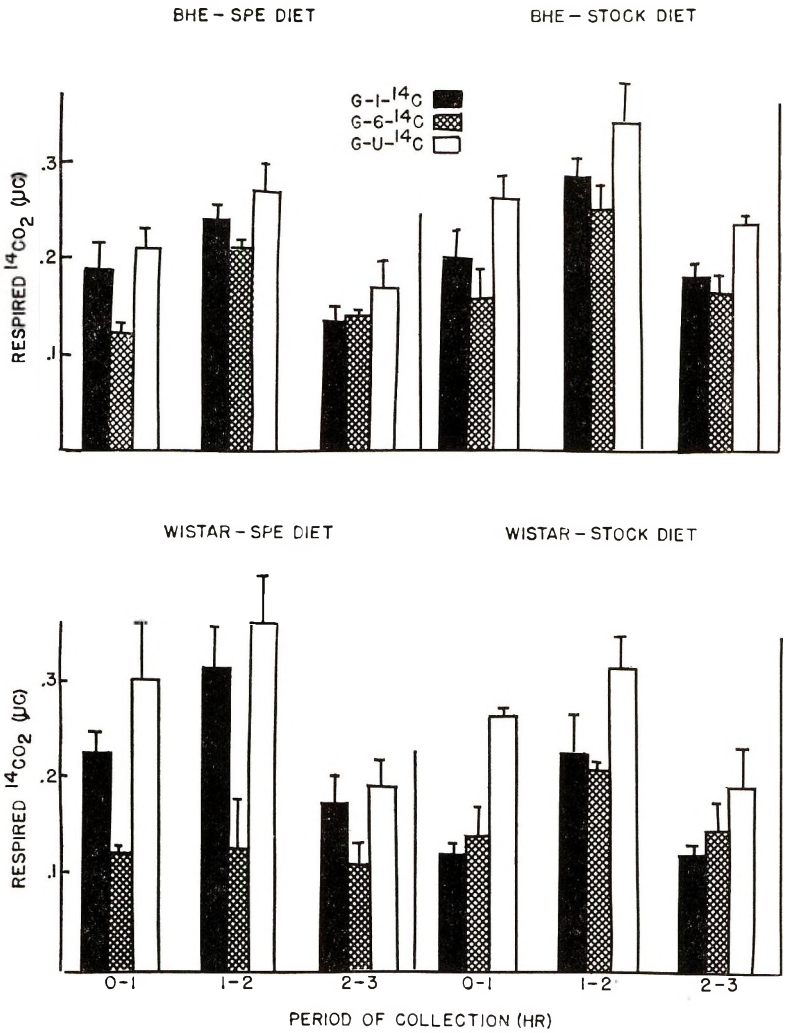

Fig. 1 Respired $^{14}CO_2$ (μCi) in three one-hour collections following the ingestions of 2 μCi of glucose-1-^{14}C, glucose-6-^{14}C or glucose-U-^{14}C (calculated from 4 μCi) of BHE and Wistar rats fed the SPE or stock diet. Each value represents the mean of the values for four rats with the standard error of the mean.

tion, the Wistar rats fed the SPE diet had the greatest activity in this metabolic system. In contrast, G-6-PD in the livers of this group had the lowest activity. Apparently the activity of this enzyme in the liver is not an indicator of the total amount of glucose being oxidized in the body by this enzyme. The activity of this enzyme in the kidney did not differ with either diet or strain. The possibility, however, that other tissues might have very different activities has not been excluded.

Liver G-6-Pase activity which is the major source of blood glucose in the fasted animal was higher in rats fed the SPE diet than in rats fed the stock diet. It is consistent with the report that dietary sucrose produced an elevated activity of this enzyme (15, 16).

Liver aldolase activity which functions in both glycolysis and gluconeogenesis was elevated in both strains when the SPE diet was fed, although this enzyme is not believed to be rate limiting for either glycoly-

sis or gluconeogenesis in the liver. Other studies of this enzyme have not indicated that it is an adaptive enzyme (17). It apparently can be significantly modified by diet, however, and further study is needed to learn if there has been a general adaptation of the enzymes in either of these metabolic pathways.

β-Glucuronidase is found in high levels in the lysosomes. The marked differences in activities between the strains suggest differences in these organelles between the strains. Histological studies using the light microscope (18) have not detected obvious differences. However, more detailed studies will be made. Studies with the electron microscope are underway.

This survey of enzyme activities from different metabolic pathways shows some of the problems to be encountered if the basic metabolic differences between these two strains and their responses to diet are to be worked out through studies of enzyme activities. More differences than similarities in the enzyme activities investigated were found between the two strains. When other enzymes are studied we can expect to find other differences. The problems of relating these differences to metabolic responses, or to differences in disease susceptibility between the strains and diets, will prove difficult. Clearly many types of information are needed. Only when such information is available will conclusions be justified.

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Effect of Undernutrition on the Size and Composition of the Rat Brain [1,2]

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ABSTRACT This study was designed to determine the effects of various periods of undernutrition on the growth and composition of the rat brain. Values for body weight, brain weight and total DNA, RNA, lipid and protein in the brains of rats on restricted feed consumption from 5 until 11, 17 and 60 days of age were significantly lower than the values for age-matched controls in each case. Also, the total cholesterol, phospholipid and cerebroside content of the brains of rats on restricted feeding until 60 days of age was lower than values obtained for brains of age-matched controls. Animals on restricted feed intake until at least 17 days of age did not recover any brain DNA or RNA when fed ad libitum until 110 days of age. Animals on restricted feed intake until 11, 17 or 60 days of age, then fed ad libitum until 110 days of age, did partially recover their deficit in brain weight and total brain protein and lipid, but these values were still significantly lower than normal. Also, the percentage of lipid in all of these brains remained significantly lower than normal. Similarly, the total amount and the percentage of phospholipid, cholesterol and cerebroside remained significantly lower than normal in the brains of rats undernourished until 60 days of age then fed ad libitum from 60 until 110 days of age. Of three major brain regions examined, restricted feeding affected the weight and DNA content of the cerebellum most severely.

Undernutrition during infancy and childhood is a prevalent problem in many parts of the world, but very little information is available on the effect of this undernutrition on brain growth and composition. Animal studies provide a reasonable means of obtaining information relevant to this problem, and data from such studies will undoubtedly aid in the understanding of the effect of undernutrition on the brain growth and composition of children.

By the age of weaning, the rat brain has become relatively resistant to undernutrition. For instance, by 16 days of age the rat brain has its full adult complement of DNA and RNA (1), and even severe caloric restriction begun as early as 3 weeks of age (2) or during the adult life (3) of the rat has no effect on brain DNA content. Also, other rat brain components are largely resistant to the effect of undernutrition initiated during the postweaning period. Dobbing and Widdowson (4) noted that brain size and phospholipid and cholesterol content were lowered by undernutrition begun at weaning, but were readily restored to normal by subsequent ad libitum feeding.

There is evidence that brain size and composition are more readily affected by undernutrition during the preweaning period than during the postweaning period. Winick and Noble (2) reported that undernutrition of rats from birth until 21 days of age resulted in lower brain weight and DNA, RNA and protein content, and subsequent ad libitum feeding did not eliminate these differences. Culley and Mertz (5) noted that undernutrition of rats from 5 until 20 days of age caused a significant reduction in brain weight and its total content of phospholipid, cholesterol and cerebroside. Recently, Guthrie and Brown (6) found that undernutrition of rats during the preweaning period caused irreversible decrements in brain size and DNA content. Brain cholesterol level was irreversibly lowered only if undernutrition was continued until five weeks of age, and brain RNA and phospholipid levels were not affected. Nevertheless, not all workers have found that undernutrition during the pre-

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weaning period affects brain size and composition. Rajalakshmi et al. (7) found that undernutrition from birth until 28 days of age did not affect rat brain size or its DNA, RNA or protein content. Benton et al. (8) found that the effects of undernutrition on rat brain size and its phospholipid, cerebroside and cholesterol content could be overcome by ad libitum feeding from 3 until 6 weeks of age.

The purpose of this study was a) to determine the effect of varying periods of undernutrition on rat brain DNA, RNA, protein and lipid content during the period of restricted feed intake, as well as after a recovery period during which they are fed ad libitum, and b) to determine whether any deviations noted in brain weight and DNA content affect three major brain regions to an equal extent.

METHODS

At 5 days of age rats of the Wistar strain were assigned either to a control litter and left with their dam at all times until weaned or to an experimental litter, in which case the young were left with their dam one time each day, only long enough for each animal to gain about 0.5 g/day. This usually required between 2 and 4 hours. The mean weight of all animals was 11 g at 5 days of age. The mean weight of experimental animals was 22 g when weaned at 23 days of age. These weaned experimental animals were then maintained at that weight by limiting feed consumption until killed or until started with ad libitum feeding. All control litters were weaned at 20–21 days of age and fed ad libitum until killed. The mean weight of control animals was 45 g when weaned. The feed consumed by all rats consisted of the following: (in percent) casein, 30; dextrose, 57.7; salt mix, USP XIV, 5; cellulose, 2; DL-methionine, 0.3; and cottonseed oil, 5. The each kilogram of this feed, we added the following vitamins: (in milligrams) thiamine·HCl, 15; riboflavin, 10; pyridoxine·HCl, 5; choline chloride, 2000; niacin, 100; calcium pantothenate, 40; *p*-aminobenzoic acid, 100; inositol, 250; folic acid, 2; biotin, 0.25; vitamin B_{12}, 0.12; menadione, 20; vitamin D_2, 6; α-tocopherol, 250; and vitamin A palmitate, 125.

At the appropriate age each animal was anesthetized with diethyl ether; the skull was opened, the olfactory lobes were dissected away, and the whole brain anterior to the foramen magnum was removed and weighed. In one experiment each brain was dissected into the cerebellum, pons-medulla and mid-plus forebrain. Two brains or brain regions were then combined, homogenized in 19 volumes of chloroform–methanol (2:1) and filtered through a medium porosity scintered-glass filter. An aliquot of the lipid filtrate was placed in a small beaker and dried at 105° to constant weight for calculation of total brain lipids. Portions of the remainder of the lipid filtrate were washed according to Folch et al. (9), analyzed for cholesterol (10) and hexose (11), and digested and analyzed for phosphorus (12, 13). Phospholipid was calculated by multiplying the phosphorus value by 25. Cerebroside plus cerebroside sulfate was calculated by multiplying the hexose value by 4.55. The nonlipid residue from two brains was washed one time with 4 ml of ice-cold ethanol, four times with 4-ml portions of ice-cold 5% trichloroacetic acid, one time with ice-cold water–ethanol (1:1), and two times with 4-ml portions of ethanol–diethyl ether (1:3). Each residue was then placed in 40 ml of 5% trichloroacetic acid and heated in an oil bath for 30 minutes at 92° with occasional agitation (14). After cooling, the nucleic acid extracts were filtered, and portions of the filtrate were analyzed for DNA using diphenylamine (15) and for RNA using *p*-bromophenylhydrazine (16). The residue was analyzed for protein nitrogen by the micro-Kjeldahl method (17).

RESULTS

The total DNA per rat brain was similar for rats fed ad libitum at 17, 60 and 110 days (A17, A60 and A110) of age, but the level at 11 days of age (A11) was only about 70% of the adult level (table 1). Brains from rats on the restricted feeding regimen until 11, 17 and 60 days (R11, R17 and R60) each contained significantly less DNA and RNA than controls of the same age ($P < 0.01$).

Also, the R60-rat brain contained essentially the same amount of DNA as the R17

TABLE 1

Effect of feed restriction on the composition of rat brain

Regimen [1]	A11	A17	A60	A110	R11	R11A110	R17	R17A110	R60	R60A110
No. of rats	12	10	12	12	10	10	10	10	12	12
Body wt, g	23 ± 2	37 ± 3	247 ± 25	428 ± 54	13 ± 1	392 ± 48	16 ± 1	321 ± 45	24 ± 1	289 ± 40
Brain wt, mg	1021 ± 41	1390 ± 57	1823 ± 37	2083 ± 105	801 ± 32	1841 ± 98	1010 ± 36	1708 ± 87	1102 ± 53	1672 ± 72
DNA, mg/brain	1.77 ± 0.10 [2]	2.46 ± 0.09	2.56 ± 0.08	2.52 ± 0.11	1.35 ± 0.07	2.29 ± 0.11	2.09 ± 0.09	2.15 ± 0.09	2.12 ± 0.09	2.07 ± 0.12
DNA, % [3]	0.173 ± 0.011	0.177 ± 0.008	0.140 ± 0.005	0.121 ± 0.007	0.169 ± 0.005	0.124 ± 0.006	0.207 ± 0.010	0.126 ± 0.006	0.192 ± 0.009	0.124 ± 0.007
RNA, %	0.259 ± 0.011	0.274 ± 0.013	0.180 ± 0.008	0.162 ± 0.010	0.281 ± 0.015	0.157 ± 0.008	0.321 ± 0.019	0.163 ± 0.008	0.230 ± 0.012	0.159 ± 0.009
Lipid, %	5.8 ± 0.2	7.0 ± 0.3	10.6 ± 0.3	11.8 ± 0.3	5.3 ± 0.2	11.2 ± 0.4	6.2 ± 0.2	10.7 ± 0.3	9.4 ± 0.3	10.5 ± 0.4
Protein N, %	1.12 ± 0.06	1.30 ± 0.08	1.74 ± 0.07	1.85 ± 0.09	1.14 ± 0.07	1.87 ± 0.07	1.26 ± 0.06	1.84 ± 0.07	1.69 ± 0.08	1.82 ± 0.09

[1] A11, A17, A60 and A110 indicate animals fed ad libitum from birth until killed at 11, 17, 60 and 110 days, respectively; R11, R17 and R60 indicate animals fed limited amounts from 5 days of age until killed at 11, 17 and 60 days respectively; R11A110, R17A110 and R60A110 indicate animals fed limited amounts (similar to R11, R17 and R60) from 5 days of age until 11, 17 and 60 days of age, respectively, and then fed ad libitum until 110 days of age.

[2] Mean ± SD for five or six samples (two brains per sample).

[3] Percentage of the component in brain.

brains, indicating that it is during the first 17 postnatal days (when the brain is ordinarily still accumulating DNA) that feed restriction can cause a low brain DNA level. Of particular interest is the fact that the lower amount of brain DNA in R17 and R60 rats could not be increased at all by ad libitum feeding until 110 days of age (R17A110 and R60A110), even though there was a greater than 50% increase in brain weight during this ad libitum-feeding period. The R17A110 and R60A110 rats had significantly less DNA and RNA per brain than A110 rats ($P < 0.01$). If ad libitum feeding was begun at 11 days of age (prior to the time the brain stops accumulating DNA), some of the deficit in DNA was overcome. As indicated in table 1, the brains of rats on restricted intake until 11 days (R11) contained only 1.35 mg of DNA, but if rats were allowed to eat ad libitum until 110 days (R11A110) the level increased to 2.29 mg. This level of DNA, however, was still lower ($P < 0.01$) than the level of 2.52 mg found for rats fed ad libitum from birth to 110 days of age (A110).

Restricted feeding lowers the brain RNA level such that the RNA-to-DNA ratio is comparable to the level in animals of the same age fed ad libitum. For 11- and 17-day-old rats the mean ratio of RNA to DNA was 1.56, and for 60- and 110-day-old rats it was significantly lower ($P < 0.01$), namely, 1.28. We have found that 1.56 is a typical RNA-to-DNA ratio for rat brain until approximately 20–25 days of age, at which time it begins to decrease because of a decrease in RNA. A lower and relatively constant ratio is attained at about 40–50 days of age. Similar changes in the RNA levels of cerebral cortex with age have been noted by others (18).

The percentage of protein nitrogen in the brain of restricted rats at 11, 17 and 60 days of age was not significantly different from that of controls of the same age. By utilizing the data from table 1, however, it can be shown that the brains from R17 and R60 animals contained significantly less ($P < 0.01$) protein nitrogen per unit of DNA (or per cell) than brains of controls of the same age, A17 and A60. Also, the brains of R11, R17 and R60 rats contained significantly less ($P < 0.01$) total protein nitrogen than the brains from controls of the same ages. Subsequent feeding ad libitum until 110 days of age allowed the ratio of protein nitrogen to DNA to return to a normal value for that age, but the total protein nitrogen per brain remained significantly lower ($P < 0.01$) than normal.

The brains of R11, R17 and R60 animals contained a significantly lower percentage, as well as total amount, of lipid than the brains of animals fed ad libitum of the same age, respectively, A11, A17 and A60 (table 1). Even after subsequent feeding ad libitum until 110 days of age (R11A110, R17A110 and R60A110), the percentage and total amount of brain lipid remained significantly lower ($P < 0.01$) than noted for control brains (A110). During the period of feed restriction the deficit in brain lipids per unit of DNA was very large. Subsequent feed ad libitum allowed considerable recovery of lipid, but the milligrams of lipid per milligram of DNA was still significantly lower ($P < 0.01$) than normal by 110 days of age (table 1). The brains of R60 and R60A110 rats contained significantly lower percentages of phospholipid ($P < 0.05$), cholesterol ($P < 0.01$) and cerebroside ($P < 0.01$) than rats fed ad libitum of the same ages, respectively, A60 and A110 (table 2).

Of the three major brain regions examined, restricted feeding affected the cerebellum most severely with regard to brain weight and DNA content (table 3).

DISCUSSION

Our animals on restricted feed intake had significantly less DNA and RNA per brain than controls of the same age in all experiments. When expressed as milligrams per gram of brain, however, the brain DNA and RNA levels of the animals on restricted feeding until 17 or 60 days of age were actually significantly higher ($P < 0.01$) than the controls, indicating that the feed restriction was limiting the accumulation of other major brain components more severely than DNA and RNA. When rats on restricted feed intake were subsequently fed ad libitum until 110 days of age, their brains accumulated considerable amounts of components other than DNA, which did not change at all, resulting in DNA-to-brain

TABLE 2

Effect of feed restriction on various brain lipid fractions

Regimen [1]	A60	R60	A110	R60A110
Phospholipid, %	5.55 ± 0.31 [2]	5.24 ± 0.22	5.90 ± 0.24	5.62 ± 0.19
Cholesterol, %	2.11 ± 0.09	1.86 ± 0.08	2.30 ± 0.08	1.96 ± 0.10
Cerebroside, %	1.77 ± 0.08	1.32 ± 0.10	2.03 ± 0.09	1.54 ± 0.15

[1] See table 1 for explanation of regimens. There were 12 rats on each regimen.
[2] All values represent the percentage of that component in brain. Two brains were combined for analyses. Consequently each value represents the mean ± SD for six samples.

TABLE 3

Effect of feed restriction on the DNA content of three brain regions

Regimen [1]	A110	R17A110
No. of animals	12	12
Cerebellum wt, mg	271 ± 13	216 ± 16 (−20%) [2]
DNA, mg/cerebellum	1.30 ± 0.09 [3]	0.99 ± 0.05 (−24%)
Pons-medulla wt, mg	317 ± 16	283 ± 21 (−11%)
DNA, mg/pons-medulla	0.273 ± 0.016	0.254 ± 0.071 (−7%)
Mid- and forebrain wt, mg	1345 ± 42	1161 ± 58 (−14%)
DNA, mg/mid- and forebrain	1.03 ± 0.08	0.88 ± 0.04 (−15%)

[1] See table 1 for explanation of regimens.
[2] Values in parentheses represent the percentage by which the R17A110 value is less than the corresponding A110 value.
[3] Brains were analyzed in pairs. Consequently each value represents the mean ± SD for six analyses.

weight ratios comparable to controls of the same age.

Our previous findings (5), as well as those presented here, are in accord with the report of Benton et al. (8) that feed restriction during the preweaning period results in deficits in brain weight and brain lipid, phospholipid, cholesterol and cerebroside content. They (8) observed, however, that when initially deprived rats were fed ad libitum from 21 until 42 days of age, their brain weight and the brain content of each of these lipids returned to normal. In contrast, our animals on restricted feeding until 11, 17 and 60 days never attained normal brain weight or lipid content, even after feeding ad libitum until 110 days of age. During the time of feed restriction, their (8) animals gained 1.0 to 1.5 g/day whereas our animals were allowed to gain only 0.5 g/day. This difference in the degree of undernutrition may explain why the brains of our animals were irreversibly altered in size and composition and the brains of their animals were not.

Our data on brain DNA and RNA are basically in agreement with that of Winick and Noble (2, 19) who found that undernutrition of rats from birth until 3 weeks of age caused a significant deficit in brain DNA and RNA that was not overcome by subsequent feeding ad libitum until 19 weeks of age. The brains of their 3-week-old control and experimental animals, however, contained only about 70% as much DNA as 19-week-old control and experimental animals, indicating that brain DNA was still accumulating at 3 weeks of age when feeding ad libitum was begun. The brain DNA content of our 17-day-old controls was not significantly different from that of 60- or 110-day-old controls. Similarly, the brains of animals on restricted feeding until 17 days of age, even though their DNA content was about 20% below control values, were unable to accumulate any additional DNA during subsequent feeding ad libitum until 110 days of age. Our animals on restricted feeding for 6 days (from 5 to 11 days of age) did recover some brain DNA during subsequent ad libitum feeding, but the brain DNA content at 110 days of age was significantly less than control values. Guthrie and Brown (6) also found that feed restriction from birth until 3 weeks of age caused an irreversible decrement in brain DNA but, in contrast to our data and that

of Winick and Noble (2), did not find a significant reduction in brain RNA. Undernutrition of young pigs (20) and mice (21) also results in permanent decrements in brain DNA.

In our experiments (tables 1 and 2) the total amount and the percentage of brain lipid, phospholipid, cholesterol and cerebroside of animals on restricted feeding, were not restored to normal by subsequent ad libitum feeding. In each case feed restriction was initiated at 5 days of age. Guthrie and Brown (6) found that undernutrition from birth until at least 5 weeks of age caused a reduction in total brain cholesterol that could not be overcome by subsequent ad libitum feeding. However, the percentage of cholesterol in the brains of the animals was normal. They found no permanent effect on brain phospholipid content and did not report values for brain lipid or cerebroside content. Although it is difficult to determine the reason that our phospholipid values and, to a lesser extent, our cholesterol values are at variance with those of Guthrie and Brown (6), it is possible that the feeding regimen or diet composition could be relevant factors. For instance, they fed ad libitum amounts of low protein diets to their experimental animals, and we fed limited amounts of a high protein diet to our experimental animals. Also, during the preweaning period their experimental animals were with their dams at all times, and our experimental animals were with their dams for only a few hours each day, resulting in a difference in feeding frequency.

Dobbing and Widdowson (4) found that undernutrition initiated at 3 weeks of age caused lower brain cholesterol and phospholipid values, but this effect could be totally overcome by subsequent ad libitum feeding. Thus, it appears that undernutrition initiated during the preweaning period causes an irreversible deficit in brain lipid, as shown by our data and that of Guthrie and Brown (6), whereas undernutrition initiated during the postweaning period results in a deficit in brain lipid that can be corrected by ad libitum feeding (4).

Continuing feed restriction from 17 until 60 days of age prolongs the diminished rate of accumulation of protein and lipids by the brain. The brains of our animals fed ad libitum accumulated 96 mg of lipid and 13.6 mg of protein nitrogen between 17 and 60 days of age, whereas the rats on restricted feed consumption accumulated only 41 mg of lipid and 5.9 mg of protein nitrogen during that same period. However, R17A110 and R60A110 brains did not differ significantly in weight or in protein or lipid content, indicating that feed restriction beyond 17 days of age had no effect on the brain weight or brain lipid and protein content of rats subsequently fed ad libitum until 110 days of age. The brains of animals fed ad libitum from 11 days until 110 days of age (R11A110), however, are larger and contain more protein and lipid than the brains of R17A110 and R60A110 rats. These data indicate that the ability of the rat brain to recover from feed restriction, at least with regard to brain weight and DNA, RNA, protein and lipid content, is established later than 11 days of age but not later than 17 days of age. This coincides with the age that the rat attains its adult complement of DNA (or cells) and suggests that the ability of the rat brain to recover from feed restriction is dependent upon the number and types of cells that it contains by 17 days of age.

Undernutrition during the postnatal period caused a deficit in brain DNA (indicating that fewer cells were present) which was greatest in the cerebellum and least in the pons-medulla (table 3). This is consistent with the report that the rate of cell formation is quite high in the cerebellum and low in the medulla during the postnatal period (22). Only glia (22, 23) and short-axoned granule cells or microneurons (22) are formed postnatally. Long-axoned nerve cells or macroneurons are formed only during the prenatal period (22) and, consequently, would not be affected by postnatal undernutrition.

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Dietary Effects on Rat Liver Enzymes in Meal-fed Rats [1]

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ABSTRACT The effects of dietary changes and starvation were studied in meal-fed, male rats of the Sprague-Dawley strain following a training period of 2 weeks and a period of protein deprivation of 4 days. A 90% casein diet increased the activities of the enzymes glucose 6-phosphatase, glucose 6-phosphate dehydrogenase, serine dehydrase, tyrosine-α-ketoglutarate transaminase, glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase 24 hours after the meal. Serine dehydrase and glutamic-oxaloacetic transaminase activities were further increased in animals killed 48 hours after the protein meal. An increase in the activity of serine dehydrase was also observed in rats killed 48 hours after their last protein-free meal. The feeding of diets which contained both carbohydrate (either glucose or fructose) and casein, increased glucose 6-phosphate dehydrogenase and malic enzyme activities to a greater extent than the 90% casein diet, but the increases in transaminase activities were less than those obtained by feeding the 90% casein diet. Serine dehydrase activity was not increased by a 65% carbohydrate diet and was increased only slightly by diets containing 57.5% casein plus 32.5% carbohydrate. Tyrosine-α-ketoglutarate transaminase activity was increased by this latter diet almost as well as by the 90% casein diet. The inducing effect of carbohydrates on enzyme activities was observable, even if the test diets contained considerable amounts of protein, whereas the inducing effect of protein was considerably decreased by even a moderate amount of carbohydrate.

Recently it was reported that the activities of a number of rat liver enzymes are increased by a high protein diet following a 4-day period of feeding a high carbohydrate, protein-free diet (1). Two patterns of increases were observed: in one group of enzymes, maximal activity was reached within 48 hours after the dietary change, whereas the activities of another group of enzymes increased by a sigmoidal pattern reaching a maximum in about 4 days (1). To further investigate the time-course of increases of those enzymes which reached maximum activity within 48 hours after the dietary change, a number of experiments were undertaken with rats fed ad libitum. It was found, however, that there was a considerable variation with respect to the time the animals began to eat and also in the amount of food consumed within the first 12 hours after the dietary change and, therefore, it appeared that this feeding technique was unsuitable for short-term experiments. It seemed advantageous, therefore, to perform these experiments with meal-fed rats which are trained to consume their daily food intake within a relatively short and standardized time period.

The effects of meal-feeding on rats have been studied by a number of authors (2–4). Their work suggests that this feeding technique may alter the manner in which rats respond to diets. We have, therefore, studied the effects of feeding various diets to meal-fed rats following a 4-day period of protein deprivation.

EXPERIMENTAL

Animals. Male rats of the Sprague-Dawley strain, weighing 150 to 170 g, were used in most experiments except in one case when the starting weight of animals was between 240 and 270 g. The animals were trained for a period of 2 weeks to eat their food in a feeding period of 2 hours/day. A jelled laboratory preparation was used for this training. After the 2-week training period the animals were fed 4 meals of the 90% dextrin diet, 1 meal/day. Following protein deprivation the animals were fed 1 meal of test diet.

Diets. All purified diets contained 5% corn oil, 4% P.H. salts (5) and 1% vitamins (6). The purified diets were fed in the powdered form. The jelled laboratory

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preparation was prepared by mixing 1.5 liters of boiling 4% agar solution with 1 kg of powdered laboratory ration.[2] The 90% dextrin diet (powder) contained corn oil, P.H. salt and vitamins in the same proportion as the experimental diets. Several test diets were used; a high protein diet (90% casein), two high carbohydrate diets containing 65% glucose or fructose and 25% casein and two diets with intermediate protein content of 57.5% casein and 32.5% glucose or fructose. The animals were fed in the early morning and were allowed free access to water.

Determinations. The animals were killed 24 hours after their last meal, or 48 hours after their last meal. The latter group is referred to as starved. The animals were killed by a sharp blow to the head, followed by decapitation and exsanguination. The liver was quickly removed, weighed and chilled. The preparation of liver homogenates and determination of enzyme activities were described elsewhere (1, 7, 8). Tyrosine-α-ketoglutarate transaminase activity was determined at 310 mμ (9). Liver protein was determined by a modified biuret procedure (10) and liver glycogen by the nephelometric procedure of Hansen et al. (11).

RESULTS

Adaptation to the feeding schedule. The extent of adaptation to the feeding schedule varied from group to group and also, though to a lesser extent, within groups. The animals invariably lost weight; this was particularly severe during the feeding of the 90% dextrin diet. At the beginning of the training period some animals refused to eat, but by the end of the second week of training consumption of the jelled laboratory preparation reached 15 to 25 g/meal.

Attempts were made to adapt the animals (following the 2-week training period) to a 90% glucose or 90% fructose diet in the powdered form. Even after 4 meals, however, consumption of these diets was extremely small. To avoid problems which might arise from osmotic disturbances of the stomach, these diets were fed in the jelled form prepared by mixing 1 liter of boiling 4% agar solution and 1 kg of the diet. Food consumption was improved by this technique, but it was suspected that the diets were not clearing the stomach fast enough, as considerable amounts of gel were found in the stomach of some animals 24 hours after the meal. In addition, the influence of such diets on enzyme activity was erratic and nonreproducible. It has been reported that because of the high molecular size of dextrin, osmotic difficulties in the stomach during and after carbohydrate feeding can be avoided by feeding dextrin (12). The use of these diets (90% glucose or fructose) was, therefore, discontinued and we decided to use a 90% dextrin diet in the powdered form. The animals were fed 4 meals of this diet to subject them to a 4-day period of protein deprivation. The animals accepted this diet but consumed less of it than of the jelled laboratory preparation.

The process of adapting to the feeding schedule was accompanied by very noticeable changes in behavior; rats became overactive, they would stand on their hind legs chewing on the cage and would attack any moving object within reach.

Effect of meal-feeding on food intake, body size, soluble liver protein and liver glycogen. Consumption of the jelled laboratory preparation was variable; rather small during the first few days of training and 15 to 25 g/rat/meal toward the end of the 2-week training period. Since over half of this preparation consisted of water, the 15 to 25 g of jelled preparation is equivalent to 6 to 10 g of the pelleted form of this diet. The consumption of the other diets was smaller, especially of the 90% dextrin diet, and are as follows in grams per rat per day ± standard error of the mean: 90% dextrin, 5.8 ± 1.1; 90% casein, 6.2 ± 0.9; 65% glucose, 8.0 ± 0.9; and 65% fructose, 6.8 ± 1.2. It should be noted, however, that the size of the meal of the test diets did not differ significantly from the average size of the dextrin meals and, therefore, the changes which occurred in liver enzyme activities after feeding the test diets should be attributed to the composition of the test diets rather than increased food intake.

Body size was reduced considerably during the 2-week training period and was fur-

[2] Purina Rat Chow, Ralston Purina Company, St. Louis.

TABLE 1

Effect of various test diets on soluble liver protein and liver glycogen of meal-fed rats

Diet fed	Soluble protein	Glycogen
	mg/100 g body wt	
Jelled laboratory ration		
Exp. 1 (4) [1]	496 ± 16 [2]	< 10
Exp. 2 (4)	481 ± 17	< 10
Exp. 3 (4)	397 ± 25	14 ± 4
Heavier rats (4)	368 ± 14	< 10
4 meals, 90% dextrin		
Exp. 1 (4)	437 ± 26	12 ± 2
Exp. 2 (4)	394 ± 10	12 ± 4
Exp. 3 (4)	332 ± 10	32 ± 11
Heavier rats (4)	332 ± 6.0	17 ± 4
Starved		
Exp. 1 (4)	299 ± 19	< 10
Exp. 2 (4)	312 ± 6	< 10
90% casein		
Exp. 1 (4)	520 ± 27	37 ± 3
Exp. 2 (4)	588 ± 19	41 ± 10
Heavier rats (4)	487 ± 15	31 ± 5
Starved (4)	403 ± 15	< 10
65% glucose, 25% casein		
Exp. 1 (4)	537 ± 23	30 ± 5
Exp. 2 (4)	557 ± 45	51 ± 17
Heavier rats (4)	441 ± 17	22 ± 6
32.5% glucose, 57.5% casein (8)	590 ± 29	120 ± 16
65% fructose, 25% casein (4)	491 ± 18	28 ± 9
32.5% fructose, 57.5% casein (7)	553 ± 26	133 ± 16

[1] Number of animals per group.
[2] SE of mean.

ther reduced during protein deprivation. The total weight loss equaled 20 to 40% of the orginial body weight.

Soluble liver protein was decreased during protein deprivation and was increased by the test diets (table 1). The extent of replenishment did not seem to be a function of the protein content of the test diet. A possible reason for this may be that, since the size of the meals was small, a considerable amount of the ingested food may have been used for the production of glucose.

Liver glycogen values were below the level of detection in the animals fed the jelled laboratory preparation (table 1). In the animals fed dextrin, glycogen values were still very low. These observations are consistent with earlier findings that liver glycogen is considerably depleted 24 hours after a meal (3). In the animals receiving test diets containing various amounts of casein, however, liver glycogen values were considerably higher. It was also noted that these animals had a varying amount of food in their stomach, indicating that in these animals the stomach contents were emptied at a slower rate. This may have been the reason for the higher values of liver glycogen in these animals.

Effects of meal-feeding on rat liver enzyme activities. The activity of fructose 1,6-diphosphatase was increased by the 90% casein diet and also by the 65% glucose diet (table 2). The increases, however, were very small. Pyruvate kinase activity was not increased by the 90% casein diet but was increased by the 65% glucose diet. The lack of a transitory elevation of pyruvate kinase activity in meal-fed rats after feeding a 90% casein diet indicates that either the transitory elevation

TABLE 2

Effect of meal feeding on rat liver constituents

Treatment [1]	Jelled laboratory ration	90% dextrin	90% dextrin + 1 meal, 90% casein	90% dextrin + 1 meal, 65% glucose	90% dextrin + 1 meal, 90% casein, starved
No. of animals	4	4	4	4	4
Weight at time of killing, g	151 ±16 [2]	126±16	113±19	113±9	98±7
RLS = (liver weight × 100)/ body weight	3.14 ±0.04	3.08±0.09	3.82±0.18 *	3.67±0.25	2.96±0.17
Fructose 1,6-diphosphatase	16.0 [3] ±1.02	14.7±0.71	17.2±0.78	18.1±1.49	7.48±0.52 *
Pyruvate kinase	56.9 ±4.20	95.3±6.40	90.3±4.41	128±14.6	51.7±2.75 *
L-α-Glycerophosphate dehydrogenase	186 ±5.30	183±5.40	196±10.3	222±18.0	142±11.8 *
Glucose 6-phosphate dehydrogenase	8.89 ±1.44	5.42±0.48	10.8±1.56 *	27.0±6.40 *	8.07±1.37
Malic enzyme	4.77 ±0.93	6.86±0.26	2.67±0.38 *	8.26±1.81	4.33±1.14
Serine dehydrase	3.95 ±0.55	2.53±0.89	6.72±1.13 *	3.41±1.64	15.5±0.72 *
Tyrosine-α-ketoglutarate transaminase	3.42 ±0.23	3.44±0.73	9.46±0.73 *	4.91±0.45	6.00±0.78 *
Glutamic-pyruvic transaminase	81.4 ±9.30	79.6±10.5	113±5.38 *	104±10.9	110±8.02
Glutamic-oxaloacetic transaminase	345 ±29.3	325±57.4	477±43.0 *	376±57.6	599±11.9 *

[1] Rats were trained for 14 days with jelled laboratory ration (Purina Rat Chow). Gel was prepared by mixing 1.5 liter of 4% agar solution with 1 kg of powdered laboratory ration. Those rats which received further dietary treatment were fed 4 additional meals of the 90% dextrin diet (powdered) and another meal when indicated. Animals were allowed to eat for 2 hours/day only.

[2] SE of mean.

[3] Enzyme activity is given in micromoles of substrate utilized per minute per 100 g body weight.

* Differs significantly from corresponding values in animals fed dextrin ($P < 0.05$).

observed in rats fed ad libitum (1) does not occur in meal-fed rats, or that the transitory elevation occurred before the rats were killed.

L-α-Glycerophosphate dehydrogenase activity was not changed by the dextrin diet in comparison with the jelled laboratory preparation, and was increased only by the 65% glucose diet in comparison with the dextrin diet. The activity of glucose 6-phosphate dehydrogenase was increased twofold by the 90% casein test diet and sixfold by the 65% glucose test diet. The increases were statistically significant in both cases. It should be pointed out that since the food intakes were approximately the same in the animals fed dextrin and also in the animals fed the test diets, the increase in glucose 6-phosphate dehydrogenase activity after feeding the 90% casein diet can be attributed to the refeeding of protein and not an increase in potentially available dietary glucose. The increase in the activity of this enzyme, however, was much greater when the test diet contained glucose, indicating that maximum induction of this enzyme under the conditions of the experiment requires not only protein, but also glucose. Malic enzyme activity was increased by the 90% dextrin diet in comparison with the activity of this enzyme in the animals fed the jelled laboratory preparation and was decreased by the 90% casein diet in comparison with the animals fed dextrin. The 65% glucose diet increased malic enzyme activity to the level found in animals fed dextrin. However, the effect of the 90% casein meal on malic enzyme activity was not reproducible (tables 3 and 4).

The activities of serine dehydrase and the three transaminases studied were increased by the 90% casein diet and also by the 65% glucose diet (tables 2 and 4).

TABLE 3

Dietary effects in heavier rats

Treatment [1]	Jelled laboratory ration	90% dextrin	90% dextrin + 1 meal, 90% casein	90% dextrin + 1 meal, 65% glucose
No. of animals	4	4	4	4
Weight at time of killing, g	192 ± 9 [2]	154 ± 5	168 ± 6	171 ± 11
RLS = (liver weight × 100)/ body weight	2.46 ± 0.08	2.41 ± 0.09	3.48 ± 0.08 *	3.13 ± 0.28 *
Fructose 1,6-diphosphatase	13.0 [3] ± 0.38	13.0 ± 0.97	13.2 ± 0.52	15.5 ± 1.57
Pyruvate kinase	62.7 ± 1.37	94.3 ± 8.38	110 ± 11.5	127 ± 25.4
L-α-Glycerophosphate dehydrogenase	143 ± 8.54	134 ± 6.63	181 ± 7.38 *	185 ± 17.5 *
Glucose 6-phosphate dehydrogenase	6.27 ± 0.63	4.66 ± 0.54	4.89 ± 0.09	17.1 ± 7.54
Malic enzyme	0.58 ± 0.16	3.24 ± 0.85	2.59 ± 1.13	4.63 ± 2.33
Serine dehydrase	8.02 ± 0.78	2.46 ± 0.03	6.65 ± 0.47 *	2.93 ± 0.47
Tyrosine-α-ketoglutarate transaminase	2.86 ± 0.08	3.97 ± 0.18	3.42 ± 0.41	3.55 ± 0.24
Glutamic-pyruvic transaminase	51.6 ± 2.91	49.6 ± 1.25	79.3 ± 13.3	63.9 ± 5.23 *
Glutamic-oxaloacetic transaminase	152 ± 8.30	121 ± 6.23	384 ± 17.5 *	225 ± 21.6 *

[1] The dietary treatment (i.e., diets and feeding schedule) of these animals was the same as described in footnote 1, table 2.
[2] SE of mean.
[3] Enzyme activity is given in micromoles of substrate converted per minute per 100 g body weight.
* Differs significantly from corresponding values in animals fed dextrin ($P < 0.05$).

It should be noted that increases in enzyme activities (as compared with the values in the animals fed dextrin) with serine dehydrase and tyrosine-α-ketoglutarate transaminase were smaller in the animals fed the 65% glucose diet than the corresponding increases in glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase activities. This tendency is in agreement with earlier reports that the synthesis of serine dehydrase and tyrosine-α-ketoglutarate transaminase is decreased by glucose (13, 14).

Effects of body size on the diet-induced increases in enzyme activities. It has been suggested by previous experimental work that enzyme levels, or the response of rat liver enzyme levels to dietary alterations may be affected by age and body size [3] (15). In these experiments the effects of dietary alterations were studied in heavier rats. The rats originally weighed about 240 to 270 g and lost considerable weight during the experiments (table 3). The responses of enzyme activities to dietary changes in the heavier rats were very similar to the responses in the lighter rats with some exceptions. These exceptions consisted of a lack of response in glucose 6-phosphate dehydrogenase and tyrosine-α ketoglutarate transaminase activity to the high protein diet and a reduction in the response of malic enzyme activity to the 65% glucose diet.

Effects of meal-feeding diets containing different amounts of protein, glucose and fructose. Since it appeared that the 90% casein diet was more effective in increasing the activities of the transaminases and serine dehydrase, whereas the 65% carbohydrate, 25% casein diets were more effective in increasing the activities of glucose 6-phosphate dehydrogenase and malic enzyme, the effects of diets with an intermediate protein content were examined (table 4). Although the carbohydrate content of these diets was only 32.5%, the increases in glucose 6-phosphate dehydrogenase and malic enzyme activities were not different from the increases obtained by feeding the 65% carbohydrate diets. After the 57.5% protein meal, glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase values were similar to the activities of these enzymes after feeding the 65% carbohydrate diet, whereas serine dehydrase activity was slightly higher after the 57.5% protein meal. In contrast to this, tyrosine-α-ketoglutarate transaminase activity was increased by the 57.5% casein diets almost as well as by the 90% casein diet. There is difficulty in ascertaining whether increased enzyme activity was brought about by increasing the protein content of the diet, or by decreas-

[3] Avery, E. H., and R. A. Freedland 1965 Relation of age and weight to liver enzyme activity. Amer. Zool., 5: 639 (abstract).

TABLE 4

Effects of varying the protein content of diets on liver constituents of meal-fed rats

Treatment [1]	Jelled laboratory ration	90% dextrin	90% dextrin +1 meal, 90% casein	90% dextrin +1 meal, 65% glucose, 25% casein	90% dextrin +1 meal, 65% fructose, 25% casein	90% dextrin +1 meal, 32.5% glucose, 57.5% casein	90% dextrin +1 meal, 32.5% fructose 57.5% casein
No. of animals	4	4	4	4	4	8	7
Weight at time of killing, g	132 ± 13 [2]	122 ± 8	102 ± 10	114 ± 7	112 ± 5	148 ± 8	141 ± 21
RLS = (liver weight × 100)/body weight	3.15 ± 0.14	2.95 ± 0.08	4.46 ± 0.14 *	3.93 ± 0.14 *	3.51 ± 0.12 *	4.50 ± 0.12 *	4.51 ± 0.15 *
Glucose 6-phosphatase	44.3 [3] ± 7.81	57.4 ± 3.26	90.8 ± 6.94 *	64.4 ± 3.58	74.9 ± 1.46 *	64.1 ± 3.26	80.1 ± 2.52 *
Fructose 1,6-diphosphatase	15.7 ± 1.18	12.3 ± 0.66	18.6 ± 0.66 *	18.5 ± 0.60 *	14.5 ± 0.93	20.8 ± 0.87 *	20.4 ± 0.61 *
Pyruvate kinase	50.3 ± 4.49	81.3 ± 12.3	58.3 ± 3.10	115 ± 4.80 *	84.5 ± 4.22	112 ± 13.2	129 ± 10.3
L-α-Glycerophosphate dehydrogenase	170 ± 10.2	157 ± 9.20	212 ± 8.99 *	269 ± 23.0 *	213 ± 8.10 *	239 ± 3.07 *	260 ± 17.4 *
Glucose 6-phosphate dehydrogenase	14.1 ± 2.20	7.97 ± 1.16	12.3 ± 0.73 *	20.8 ± 6.58	27.7 ± 2.73 *	24.7 ± 3.02 *	23.5 ± 2.85 *
Malic enzyme	8.67 ± 0.60	5.43 ± 1.09	8.43 ± 1.38	15.9 ± 5.50	14.6 ± 1.44 *	14.9 ± 1.31 *	14.7 ± 1.04 *
Serine dehydrase	5.63 ± 0.38	2.39 ± 0.12	6.22 ± 0.77 *	2.57 ± 0.31	2.55 ± 0.84	3.46 ± 0.75	3.10 ± 0.26 *
Tyrosine-α-ketoglutarate transaminase	4.06 ± 0.04	2.46 ± 0.03	14.6 ± 0.26 *	3.25 ± 0.30 *	2.75 ± 0.34	10.8 ± 0.88 *	14.4 ± 1.17 *
Glutamic-pyruvic transaminase	92.6 ± 4.27	56.5 ± 3.73	122 ± 7.80 *	93.8 ± 5.91 *	89.5 ± 8.20 *	97.7 ± 8.84 *	81.1 ± 9.07 *
Glutamic-oxaloacetic transaminase	202 ± 9.30	195 ± 7.80	342 ± 11.8 *	310 ± 38.7 *	255 ± 7.25 *	293 ± 8.15 *	293 ± 15.6 *

[1] The dietary treatment (i.e., diets and feeding schedule) of these animals was the same as described in footnote 1, table 2.
[2] SE of mean.
[3] Enzyme activity is given in micromoles of substrate utilized per minute per 100 g body weight.
* Differs significantly from corresponding values in animals fed dextrin ($P < 0.05$).

ing carbohydrate content, or both. This difficulty is compounded since serine dehydrase synthesis has been shown to be inhibited by glucose at the translation level (13), yet its induction seems to require the intake of a certain amount of protein.[4] It may be possible that such controls regulate the synthesis of other enzymes as well.

It appears then that the induction of glucose 6-phosphate dehydrogenase and malic enzyme is maximum when the inducing diet contains some carbohydrate. The induction of these enzymes requires some protein in the diet and proceeds, in meal-fed rats, even if the protein content is rather high. At either extreme of dietary composition (i.e., all protein or all carbohydrate), however, the activities of these enzymes are less than maximal. In contrast to this, the induction of the transaminases is maximal with the high protein, carbohydrate-free diet and even small amounts of dietary carbohydrate can reduce induction. Similar results were obtained whether the carbohydrate component of the diet was glucose or fructose, with the exception of glucose 6-phosphatase, the activity of which was higher in rats receiving dietary fructose.

Effects of starvation of meal-fed rats. The possibility was considered that meal-feeding causes physiological alterations resembling starvation (i.e., low liver glycogen and high transaminase values) and, therefore, the effects of killing the animals 48 hours after their last meal were investigated. Two types of starvation experiments were performed. In one type (table 2) the animals were starved after receiving a 90% casein meal, and in the other type (table 5), the animals were starved after receiving their last 90% dextrin meal.

In both types of experiments, pyruvate kinase activities were sharply reduced by starvation. L-α-Glycerophosphate dehydrogenase activities were also reduced, but to a smaller extent. Both glucose 6-phosphate dehydrogenase and malic enzyme activities were reduced by starvation after dextrin feeding, but after protein feeding the activity of the former was slightly less than

[4] Harper, A. E., and G. Sando, unpublished results.

TABLE 5

Effect of starvation on rat liver constituents in meal-fed rats

Treatment [1]	Jelled laboratory ration	90% dextrin	90% dextrin starved	
			Exp. 1	Exp. 2
No. of animals	4	4	4	4
Weight at time of killing, g	132 ±4 [2]	112±3	102±9	120±11
RLS = (liver weight ×100)/ body weight	3.00 ±0.16	2.96±0.08	2.30±0.17 *	2.50±0.09 *
Glucose 6-phosphatase	45.1 [3]±3.89	55.4±1.00	46.6±4.43	59.4±7.92
Fructose 1,6-diphosphatase	12.6 ±0.45	11.2±0.68	10.4±0.35	9.05±1.24
Pyruvate kinase	43.8 ±3.77	57.0±6.60	39.0±1.81 *	48.8±5.90
L-α-Glycerophosphate dehydrogenase	141 ±9.68	134±5.88	112±10.2	104±14.9
Glucose 6-phosphate dehydrogenase	7.69 ±1.75	4.37±0.79	5.99±0.48	3.69±0.35
Malic enzyme	3.67 ±0.71	5.25±0.87	2.93±1.21	3.54±0.56
Serine dehydrase	3.57 ±0.30	2.70±0.28	7.61±0.54 *	4.83±0.96
Tyrosine-α-ketoglutarate transaminase	1.79 ±0.12	2.18±0.52	2.39±0.34	5.53±0.41 *
Glutamic-pyruvic transaminase	85.0 ±1.84	73.1±1.42	63.5±2.04	54.4±6.30 *
Glutamic-oxaloacetic transaminase	127 ±14.7	144±17.3	194±24.5	138±18.1

[1] The dietary treatment of these animals was the same as described in footnote 1, table 2. The exception is that the animals designated as "starved" were killed 48 hours after their last meal.
[2] SE of mean.
[3] Enzyme activity is given in micromoles of substrate utilized per minute per 100 g body weight.
* Differs significantly from corresponding values in animals fed dextrin ($P < 0.05$).

24 hours after induction by the protein diet, whereas the activity of the latter was slightly higher than 24 hours after the protein meal.

Serine dehydrase activity was increased in both types of starvation, but glutamic-oxaloacetic transaminase values were increased only in the rats fed protein. Tyrosine-α-ketoglutarate transaminase activity was lower in animals fed protein 48 hours after the protein meal than 24 hours after the meal, but the activity of this enzyme 48 hours after the protein meal was still higher than in the animals fed dextrin. Interestingly, glutamic-pyruvic transaminase values were not increased in either of the two types of starvation experiments, and may in fact have been decreased in the starved animals previously fed the 90% dextrin diet.

It should be noted that although the effects of starvation on transaminase and serine dehydrase activities were qualitatively similar in both types of starvation, enzyme activities were considerably higher in the animals previously fed the 90% casein diet.

Further studies on dietary effects on rat liver enzyme activities. It was of interest to see if the activities of a number of other enzymes could be changed by dietary manipulations. In particular, it was important to determine whether changes in glucose 6-phosphate dehydrogenase activities are paralleled by changes in 6-phosphogluconate dehydrogenase activities. As can be seen from table 6, the activities of phosphoglucomutase, sorbitol dehydrogenase, aldolase, 6-phosphogluconate dehydrogenase, pentose phosphate metabolizing enzymes, succinic dehydrogenase, fumarase and malic dehydrogenase (DPN) were not affected appreciably by dietary changes. It appears, therefore, that in meal-fed rats the activities of these enzymes are not altered by diets over the period studied. From these results it was concluded that the activities of these enzymes either do not respond to dietary changes in meal-fed rats, or that the changes can be observed only over a longer period of time.

DISCUSSION

These experiments were undertaken to evaluate the responses of rat liver enzyme activities in meal-fed rats following protein deprivation. It was found that, although, qualitatively meal-fed rats responded to dietary changes in a manner which might be deduced from work with rats fed ad libitum, there is reason to be cautious in interpreting data obtained using meal-fed rats. For example, in these experiments the animals lost a considerable amount of body weight during training and also dur-

TABLE 6

Effect of diets on enzyme activities

Treatment [1]	Jelled laboratory ration	90% dextrin	90% dextrin starved	90% dextrin +1 meal, 90% casein	90% dextrin +1 meal, 65% glucose	90% dextrin +1 meal, 65% fructose
No. of animals	5	5	5	4	4	3
RLS = (liver weight × 100)/body weight	3.09 ± 0.09	3.11 ± 0.15	3.20 ± 0.13	3.30 ± 0.24	3.01 ± 0.17	2.83 ± 0.11
Weight at time of killing, g	162 ± 6 [2]	129 ± 4	128 ± 6	136 ± 4	127 ± 6	136 ± 7
Phosphoglucomutase	40.2 [3] ± 3.48	34.2 ± 3.50	38.9 ± 4.18	41.2 ± 1.41	45.4 ± 3.47	41.7 ± 2.82
Sorbitol dehydrogenase	11.3 ± 0.97	12.4 ± 0.22	12.1 ± 0.31	15.0 ± 1.01	13.1 ± 0.86	12.5 ± 0.52
Aldolase	12.3 ± 1.12	15.8 ± 0.47	14.8 ± 0.97	23.0 ± 1.04	8.41 ± 3.13	15.5 ± 1.05
6-Phosphogluconate dehydrogenase	5.09 ± 0.38	4.32 ± 0.15	4.75 ± 0.35	5.44 ± 0.21	5.38 ± 0.73	4.68 ± 0.35
Pentose phosphate metabolizing enzymes	6.02 ± 0.82	6.95 ± 0.57	5.23 ± 0.50	6.97 ± 0.91	6.76 ± 0.61	6.24 ± 0.74
Succinic dehydrogenase	4.16 ± 0.38	3.70 ± 0.16	3.64 ± 0.27	2.99 ± 0.11	2.70 ± 0.25	2.40 ± 0.10
Fumarase, water, extract (total)	70.5 ± 7.00	71.6 ± 4.60	71.8 ± 4.95	80.9 ± 11.7	— [4]	87.4 ± 6.62
Malic dehydrogenase, water extract (total)	786 ± 45.0	805 ± 101	629 ± 52.0	698 ± 51.0	653 ± 42.0	— [4]

[1] The dietary treatment (i.e., diets and feeding schedule) of these animals was the same as described in footnote 1, table 2.
[2] SE of mean.
[3] Enzyme activity is given in micromoles of substrate utilized per minute per 100 g body weight.
[4] Experimental difficulties prevented determination of these values.

ing the period of protein deprivation. Furthermore, food intakes were low.

The activities of glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase were increased considerably 24 hours after the feeding of a high protein meal. It may be possible, therefore, to study the early time course of induction of these enzymes with the use of antibiotics, which would not be possible with rats fed ad libitum. This technique, however, may not be suitable for studying the early time course of increases in the activities of pyruvate kinase, malic enzyme and L-α-glycerophosphate dehydrogenase after feeding a 90% casein meal, since the increases in enzyme activities may be too small for the accurate determination of rates of increases. The responses of these enzymes to a test diet containing carbohydrate may allow the study of involvement of protein and carbohydrate as possible agents of translational and transcriptional control, since the extent of increases in enzyme activities in this dietary change is large enough to allow rigorous tests of differences in enzyme activities caused by various factors. This may be especially true in the case of glucose 6-phosphate dehydrogenase and, to a lesser extent, malic enzyme.

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Influence of Microbiota on Metabolic Fecal Nitrogen in Rats

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ABSTRACT The influence of microbial flora on metabolic fecal nitrogen was determined with selected levels of protein up to 21.5%, fed as liquid and dry diets. Metabolic fecal nitrogen was less in axenic than in conventional rats when fed autoclaved dry or filtered liquid diets. Slopes of regression curves describing fecal nitrogen excretion were similar for axenic and conventional rats fed autoclaved diets, and much greater than in conventional rats on the nonautoclaved diets. The intercepts were similar for conventional rats on both nonautoclaved and autoclaved diets.

Metabolic fecal products originate from endogenous catabolism, endogenous secretions and sloughed intestinal tissue. Endogenous secretory nitrogen is derived from unabsorbed bile and from unabsorbed gastric, intestinal and pancreatic juice (1). Other products that enter the intestinal lumen and contribute to metabolic fecal nitrogen include urea [1] and coproantibodies (2, 3). Leblond and Stevens (4) observed histological changes and migration of intestinal epithelium in rats and estimated that the average life span of epithelial cells in the duodenum was only 1.57 days. Twombly and Meyer (5) calculated that the nitrogenous fecal excretions of conventional rats receiving a protein-free diet were equivalent to the nitrogen normally consumed in a 10% protein diet.

Observations cited on quantities of metabolic fecal material from epithelial tissue, digestive secretions and antibody material were made with animals containing a conventional intestinal flora. It is well known that much of the material that contributes to metabolic fecal nitrogen is ultimately incorporated into bacterial cells and excreted from the gut as such (6). Nasset (7) and Gouwens [2] observed similar fecal amino acid patterns from widely different dietary amino acid patterns. They suggested that the similarity of fecal amino acid pattern resulted from either endogenous protein secretions or microbial synthesis.

The purpose of this investigation was to determine the influence of intestinal bacteria on the quantity of metabolic nitrogen excreted as feces. Conventional and axenic rats were used in the study.

PROCEDURE

Nitrogen balance was determined in growing axenic and conventional rats fed different levels of protein. Weanling male rats of the Charles River strain, of similar genetic backgrounds, were kept in metabolism cages located in animal quarters maintained at 25°. Rats were assigned randomly to treatment from outcome groups formed on the basis of initial weight within each environment. Axenic environment was maintained with flexible plastic isolators (8). Isolators were monitored for bacterial contamination initially, at the time of supply transfers and at the termination of the trials. Diet, excreta and anal swab samples were plated on tryptose, blood and thioglycollate media and incubated at temperatures of 25 or 37°. Gram stains were also prepared from the samples.

In experiments 1 and 2, animals were fed diets containing 0.42, 4.97, 9.94 and 14.91% crude protein (dried egg white) (table 1). Rats within an outcome group were fed equally, the intake determined by the rat that would consume the least. Eight conventional rats received the nonautoclaved diets. Eight axenic and 8 conventional rats received the diets after autoclaving for 9 and 11 minutes for experiments

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[1] Harmon, B. G., D. E. Becker and A. H. Jensen 1967 Effect of microbial flora on nitrogen excretion products. J. Anim. Sci., 26: 907 (abstract).

[2] Gouwens, D. W. 1966 Influence of dietary protein and fiber on fecal amino acid excretion of swine. M.S. Thesis, University of Illinois, Urbana, Illinois.

TABLE 1

Basal diet composition (exps. 1 and 2)

Ingredient	%
Starch [1]	71.0
Cellulose [2]	2.0
Corn oil	10.0
Mineral mixture [3]	4.0
Vitamin mixture [4]	10.0
Cod liver oil	1.5
Wheat germ oil	0.5
Sodium chloride	1.0

[1] Spray-dried egg white substituted for starch to provide dietary protein levels of 0.42 (zero substitution), 4.97, 9.94 and 14.91%.
[2] Solka Floc, Brown Paper Company, Chicago.
[3] Harmon et al. (9).
[4] Contained per kilogram of mix (g): thiamine·HCl, 0.05; riboflavin, 0.1; pyridoxine·HCl, 0.05; calcium pantothenate, 0.4; niacin, 0.2; choline chloride, 20.0; vitamin B_{12}, 0.00088; menadione, 0.02; inositol, 3; *para*-aminobenzoic acid, 0.4; biotin, 0.004; ascorbic acid, 0.4; folic acid, 0.008; and starch, 975.36712.

1 and 2, respectively, at 132° with pre- and postvacuum cycles. Fiber was added to all dry diets to facilitate grinding of the autoclaved diets. The diets were finely ground prior to feeding, and wetted at time of feeding to reduce wastage and stimulate consumption.

After a 7-day precollection period, feces and urine were collected for 7 days. In preparation for analysis the feces were dried at 41°, then ground to a powdery consistency. Nitrogen was determined by the micro-Kjeldahl method.

Eight and 12 conventional rats and 8 axenic rats were used in each of experiments 3 and 4, respectively. The liquid diets (approximately 67% water) described in table 2 were prepared, sterilized by filtration through a 0.22 μ Millipore filter, and placed in storage at 4° for the two experiments at one time. The diets for experiment 3 were stored for a brief period of time; however, diets were stored approximately 45 days before experiment 4 was begun. All rats within an outcome group were fed equally (dry matter basis) for 7-day precollection and 7-day collection periods. These diets contained 0.24, 6.22, 13.53 and 21.55% protein (by analysis); added protein was provided by hydrolyzed casein fortified with amino acids. Additional water was available at all times.

TABLE 2

Composition of diets fed [1] (exps. 3 and 4)

Ingredients	Protein, % of dry matter			
	0.24	6.22	13.53	21.55
	g	*g*	*g*	*g*
Enzymatic casein hydrolysate		33.33	66.67	100.00
2 N HCl, cm^3		4.25	8.34	12.50
L-Threonine		0.2333	0.467	0.70
L-Methionine		0.2833	0.567	0.85
L-Glycine		0.150	0.300	0.45
L-Aspartic acid		0.4166	0.833	1.25
L-Proline		0.566	1.127	1.70
Sodium L-glutamate		0.916	1.834	2.75
L-Serine		0.300	0.60	0.90
Tyrosine ethyl ester·HCl		1.666	3.34	5.00
L-Cystine ethyl ester·HCl		0.183	0.366	0.55
D-Glucone-δ-lactone	13.04	13.04	13.04	13.04
D-Ethyl linoleate	2.00	2.00	2.00	2.00
D-Tween 80 (polysorbate)	3.00	3.00	3.00	3.00
Mineral mix [2]	+	+	+	+
Vitamin mix [3]	+	+	+	+
D-Dextrose monohydrate	488.02	450.16	412.31	372.42
Dry matter (by analysis)	40.8	37.0	34.0	34.8

[1] Made up to a liter with distilled water.
[2] Mineral mix: (gram per liter of diet) magnesium oxide, 0.36; potassium hydroxide, 3.08; ferrous gluconate, 0.86; manganous acetate·$4H_2O$, 0.130; cupric acetate·H_2O, 0.008; cobaltous acetate·$4H_2O$, 0.004; zinc benzoate, 0.011; ammonium molybdate·$4H_2O$, 0.003; potassium iodide, 0.015; sodium chloride, 2.40; sodium bicarbonate, 1.75; and fructose 1,6-diphosphate monocalcium salt, 25.25.
[3] Vitamin mix: (gram per liter of diet) choline chloride, 1.25; thiamine·HCl, 0.0025; riboflavin, 0.0037; pyridoxine·HCl, 0.0031; niacin, 0.0187; inositol, 0.125; calcium pantothenate, 0.025; biotin, 0.0002; folic acid, 0.0003; ascorbic acid, 0.250; vitamin B_{12} with mannitol (1% trituration), 0.05; *para*-aminobenzoic acid, 0.15; vitamin A acetate, 0.005; α-tocopherol, 0.025; menadione, 0.0021; and calciferol, 3.5 μg/liter.

TABLE 3

Fecal nitrogen (mg) per unit (g) dry matter intake for axenic and conventional rats [1]

Item	Extrapolated value at 0% protein	Dietary protein			
		0.24%	4.97%	9.94%	14.91%
Conventional rats					
Nonautoclaved					
Exp. 1 *	1.11	1.20 ± 0.30 [2]	1.29 ± 0.01	1.56 ± 0.23	1.92 ± 0.21
Exp. 2 *	1.71	1.74 ± 0.13	1.99 ± 0.04	2.53 ± 0.06	2.72 ± 0.14
Autoclaved					
Exp. 1 *	1.16	1.96 ± 0.01	2.33 ± 0.15	5.51 ± 0.90	8.01 ± 0.71
Exp. 2 *	1.69	2.07 ± 0.21	5.16 ± 0.25	9.58 ± 0.03	13.25 ± 0.06
Axenic rats					
Exp. 1 *	0.02 **	0.56 ± 0.08 **	1.66 ± 0.41	4.27 ± 0.76	7.14 ± 0.75
Exp. 2 *	0.24 **	1.00 ± 0.11 **	2.69 ± 0.12	5.25 ± 0.16	9.79 ± 0.07

[1] Dry diet. Average initial weight: experiment 1, 44 g; experiment 2, 54 g. Two rats per treatment.
[2] SE.
* Linear ($P < 0.01$) increase with increase in dietary protein.
** Less ($P < 0.01$) than values for conventional rats.

TABLE 4

Urinary nitrogen (mg) per unit of feed intake by axenic and conventional rats [1]

Item	Dietary protein			
	0.24%	4.97%	9.94%	14.91%
Conventional rats				
Nonautoclaved diet				
Exp. 1 *	1.42 ± 0.17	1.72 ± 0.55	3.01 ± 0.21	4.91 ± 0.72
Exp. 2 *	2.80 ± 0.31	2.86 ± 0.27	5.84 ± 0.33	10.52 ± 0.85
Autoclaved diet				
Exp. 1 *	3.31 ± 0.45	3.08 ± 0.71	3.69 ± 0.41	6.21 ± 0.31
Exp. 2 *	2.67 ± 0.63	4.47 ± 0.91	5.61 ± 0.44	8.76 ± 0.19
Axenic rats				
Exp. 1 *	1.40 ± 0.23	1.83 ± 0.07	2.16 ± 0.26	3.62 ± 0.02
Exp. 2 *	2.30 ± 0.15	4.51 ± 0.09	10.23 ± 0.46	10.27 ± 1.03

[1] Dry diet. Average initial weight: experiment 1, 44 g; experiment 2, 54 g.
[2] SE.
* Linear ($P < 0.01$) increase with increase in dietary protein.

RESULTS

Experiments 1 and 2. The influence of microbiota on metabolic fecal and urinary nitrogen of rats is shown in tables 3 and 4. In each environment, fecal and urinary nitrogen increased linearly (< 0.01) as dietary protein increased. The fecal nitrogen of rats fed the 0.42% protein diet, or the value derived by extrapolating to zero protein intake, was significantly ($P < 0.01$) less for axenic than conventional rats. The regression equation coefficients describing fecal nitrogen for axenic rats approximated those for conventional rats receiving autoclaved diets (table 5). Fecal nitrogen of conventional rats on autoclaved diets was significantly ($P < 0.01$) greater, and therefore, protein digestibility was lower for rats receiving 0.42% and greater protein levels, than those receiving nonautoclaved diets. Within an experiment the metabolic fecal nitrogen (MFN) (determined by extrapolation to 0% protein) was almost the same for conventional rats receiving either autoclaved or nonautoclaved diets. The slope of the regression equation, however, was greater for the rats on autoclaved diets. The heavier rats consuming more diet in experiment 2 excreted more fecal nitrogen in all environments and at all dietary protein levels. Urinary nitrogen varied with the different environments but the effects were inconsistent between experiments (table 4).

TABLE 5

Regression equations of ratio of fecal nitrogen to dry matter consumed (Y) on protein content of the diet (X)

Treatment	Regression equation
Dry diets	
Exp. 1	
Conventional rats	
Nonautoclaved diet	$Y = 1.11 + 0.050\ X$ *
Autoclaved diet	$Y = 1.16 + 0.437\ X$ *
Axenic rats	$Y = 0.02 + 0.455\ X$ *
Exp. 2	
Conventional rats	
Nonautoclaved diet	$Y = 1.71 + 0.071\ X$ *
Autoclaved diet	$Y = 1.69 + 0.776\ X$ *
Axenic rats	$Y = 0.24 + 0.592\ X$ *
Liquid diets	
Exp. 3	
Conventional rats	$Y = 3.37 + -0.002\ X$
Axenic rats	$Y = 1.22 + 0.183\ X$ *
Exp. 4	
Conventional rats	$Y = 2.49 + 0.144\ X$ *
Axenic rats	$Y = 0.39 + 0.317\ X$ *

* Linear component ($P < 0.01$).

Experiments 3 and 4. Differences in MFN between axenic and conventional rats fed dry diets were also observed with liquid diets (table 6). In axenic rats MFN was significantly ($P < 0.01$) less on the 0.24% diet and the extrapolated zero percent protein value. In experiment 3 conventional rats excreted similar quantities of nitrogen per unit of feed intake irrespective of percent dietary protein in the filtered diet. After approximately 45 days storage at 4°, however, feeding the diets resulted (exp. 4) in significant ($P < 0.01$) linear increases in fecal nitrogen per unit of feed intake for conventional rats. In each experiment fecal nitrogen from axenic rats increased ($P < 0.01$) linearly as dietary protein increased.

Urinary nitrogen (table 7) increased linearly ($P < 0.01$) in each experiment and each environment as dietary protein increased. Microbial presence influenced increased urinary nitrogen only in experiment 4 in which diets were stored for 45 days prior to feeding.

DISCUSSION

The experiments described here demonstrated that the microbiota of the intestinal tract influenced the quantity of metabolic fecal nitrogen. The presence of a conventional microbiota was associated with increased MFN from diets fed in liquid (filtration sterilization) or dry (autoclaved) forms. The linear relationships between the ratio of fecal nitrogen to dry matter consumed and to the level of protein were previously reported by Mitchell and Bert (10).

Autoclaving the diets influenced digestion at all levels of protein in the diet (0.24, 4.97, 9.94 and 14.91%). Digestion of autoclaved diets was less in both axenic and conventional rats than digestibility of nonautoclaved diets in conventional rats.

Filtering eliminated the autoclaving effects on physical characteristics and digestibility of the diets. Further, liquid diets simplified the study of excretion of fecal nitrogen in fiber-free diets. The MFN has been reported to vary directly with fiber content of the diet (11–13).

TABLE 6

Fecal nitrogen (mg) per unit of dry matter intake (g) for axenic and conventional rats [1]

Item	Extrapolated value at 0% protein	Dietary protein			
		0.42%	6.22%	13.53%	21.55%
Conventional rats					
Exp. 3	3.37	3.38 ± 0.05 [2]	3.38 ± 0.19	3.28 ± 0.16	3.28 ± 0.03
Exp. 4 *	2.49	2.51 ± 0.11	3.38 ± 0.22	4.42 ± 0.19	3.53 ± 0.09
Axenic rats					
Exp. 3 *	1.22 **	1.49 ± 0.23 **	2.15 ± 0.08	3.59 ± 0.02	5.28 ± 0.25
Exp. 4 *	0.39 **	0.99 ± 0.07 **	1.75 ± 0.18	4.62 ± 0.23	7.43 ± 0.32

[1] All diets sterilized by filtration. Average initial weight: experiment 3, 67 g; experiment 4, 59 g. Rats per treatment: experiment 3, 8; experiment 4, 8 axenic and 12 conventional.

[2] SE.

* Linear ($P < 0.01$) increase with increase in dietary protein.

** Less ($P < 0.01$) than values for conventional rats.

TABLE 7

Urinary nitrogen (mg) per unit (g) of dry matter intake by axenic and conventional rats [1]

Item	Dietary protein			
	0.42%	6.22%	13.53%	21.55%
Conventional rats				
Exp. 3 *	2.42 ± 0.10 [2]	5.53 ± 0.06	11.13 ± 1.47	19.36 ± 0.52
Exp. 4 *	4.76 ± 0.06	8.65 ± 0.45	14.38 ± 0.34	23.00 ± 1.53
Axenic rats				
Exp. 3 *	3.35 ± 0.78	4.91 ± 0.75	9.58 ± 0.15	19.33 ± 1.39
Exp. 4 *	2.86 ± 0.51	5.88 ± 0.16	13.22 ± 0.13	19.94 ± 0.78

[1] All liquid diets sterilized by filtration. Average initial weight: experiment 3, 67 g; experiment 4, 59 g.
[2] SE.
* Linear increase with increased dietary protein ($P < 0.01$).

The results of feeding the liquid diets confirm the influence of microbiota on the metabolic fecal nitrogen observed with dry diets. The MFN obtained by extrapolating total fecal nitrogen to zero protein intake is much less for axenic than conventional rats. Regression analysis demonstrates that liquid diets containing glucose and free amino acids and stored 45 days, produced quite different results when fed to conventional rats. The reduced absorption and retention in experiment 4 probably resulted from the effects of the browning reaction on the amino acids in the glucose-containing liquid diets during storage.

Morphological studies have indicated less mucosal erosions into the intestinal contents of axenic rats than of conventional rats. Gordon and Wostmann (14) reported lower small intestine weights in axenic than in conventional rats. Abrams et al. (15) used tritiated thymidine to show that renewal rates of mucosa were lower in the absence of a microflora. A similar reduction was observed for lamina propria and Peyer's patches in axenic animals. Gordon and Bruckner-Kardoss (16), using histological procedures, showed that microbial flora augments the amount of lamina propria tissue. Further support for the thesis of reduced nitrogen from intestinal mucosa was reported by Gordon and Bruckner-Kardoss (17). Surface area of upper, middle and lower segments of the small intestine was 30% less in axenic than in conventional rats. The reduction in surface area was paralleled by a reduction in dry matter. Thus, because of reduced renewal rate of intestinal mucosa, less intestinal mucosa, less intestinal surface area and reduced inflammatory response, the quantity of MFN represented by the peripheral cells of the intestine is much lower in axenic than in conventional rats.

Levenson and Tennant (18) observed fecal nitrogen values completely different from the data reported here. These workers fed different dietary protein levels (13, 20 and 40%) and found more fecal nitrogen in axenic than in conventional rats. The sterilization procedures, and the source of protein and carbohydrate were not listed; therefore, amino acid composition, carbohydrate-amino acid interaction, or autoclaving effects on amino acid availability cannot be compared for the two experiments. They also reported that 70% of the fecal nitrogen from axenic rats and about 50% from conventional rats was filterable. The differences in filterability and solubility of excreta from axenic and conventional rats emphasize the critical design of the metabolism unit. Increased solubility of the fecal nitrogen was suggested by the work of Harmon et al.[3] which showed that the percent of total fecal nitrogen represented by urea nitrogen was greater in axenic than in conventional rats. The percent of chromatographically determined amino acids was higher in conventional rats than axenic rats, suggesting that much of the fecal nitrogen was anabolized into bacterial protein.

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In vivo and in vitro Studies of Gluconeogenesis in Meal-fed and Nibbling Rats [1]

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ABSTRACT The influence of periodicity of eating on the gluconeogenic capacity of the albino rat was studied. The ability of meal-fed (access to food limited to a single 2-hour period) and nibbling (fed ad libitum) rats to convert glycerol, pyruvate and glutamate to glucose and glycogen was studied in animals deprived of food for 22 hours. The gluconeogenic capacity of the meal-fed rat was somewhat less than that of nibbling rats. This conclusion was also supported by in vitro studies in which the rate of glucose formation from these substrates (glycerol, pyruvate and glutamate) by kidney cortex slices from meal-fed and nibbling rats was determined. Studies in vivo demonstrated that the conversion of pyruvate-2-^{14}C to glucose was about 24% lower in the fasted meal-eating rat as compared with the nibbling rat. The depression of gluconeogenesis by meal ingestion was greater in the meal-fed rat. These data are interpreted to suggest that the meal-fed rat develops the capacity to change from a gluconeogenic to a glycolytic metabolism more effectively than does the nibbling animal.

Meal eating (the ingestion of food in a single, daily 2-hour meal) presents an unnatural stress to the laboratory rat accustomed to ingesting its food in frequent small meals throughout the day (1). We have previously presented data indicating that the meal-fed rat reaches the postabsorptive state approximately 8 hours after the start of the meal, and that the muscle glycogen stores are virtually depleted within 14 hours after the start of the daily meal (2). During the subsequent 10 hours before the start of the next meal, the animal is oxidizing primarily lipid; and the glucose needed is presumably supplied through gluconeogenesis, that is, the synthesis of glucose from noncarbohydrate precursors.

The capacity for gluconeogenesis is under rather precise metabolic control and increases in conditions where exogenous glucose is inadequate to meet body needs such as in diabetes, starvation and fat feeding (3, 4). Recently it has been reported that the activity of one of the gluconeogenic enzymes varies with the feeding status of meal-fed rats, decreasing following the daily meal and returning to high values during the fasting period between meals (5). This observation suggests, as would be expected, that in the meal-fed rat gluconeogenesis is enhanced during the postabsorptive period and depressed following the ingestion of the daily meal. Thus, meal-feeding would impose a daily "cycling" of increased and depressed rates of glucose synthesis. This situation would be quite a contrast to that in the rat fed ad libitum where gluconeogenesis proceeds at a low constant rate. It might be anticipated, as a result of the daily fluctuation in the rate of gluconeogenesis, that the meal-fed rat would show some characteristic adaptive changes in glucose synthesis. This is suggested by the observation that cytoplasmic pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities are increased in liver of the meal-fed rat (6).

The experiments to be described were undertaken to determine the rates of gluconeogenesis from various substrates in the meal-fed rat during the fasting state prior to the daily meal and following the ingestion of the meal. The observed rates of glucose production were compared with those of rats fed ad libitum. The results presented show that meal feeding does not

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enhance the gluconeogenic capacity but may induce a more precise and sensitive regulation of glucose synthesis.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, weighing 250–300 g, which had been fed a commercial diet,[2] were used. The animals were divided into two groups; one had access to food from 8 AM to 10 AM only (meal-eaters), and the other was fed ad libitum (nibblers). The animals were housed in metal cages having raised wire floors and in a temperature-regulated room (21°). Water was available at all times, and a semipurified diet (7) was fed. The rats were maintained on these feeding schedules for at least 3 weeks prior to use since this period has been shown to be sufficient to induce adaptive changes (8). Body weight and food consumption data were obtained and were similar to those previously reported (9).

Gluconeogenesis was studied in vitro using the kidney cortex slice preparation described by Krebs et al. (10). Meal-fed and nibbling rats maintained without food for 22 hours were killed by decapitation; the kidneys were removed and placed in cold saline. Cortex slices (approximately 100 mg) were prepared with a Stadie-Riggs hand microtome, weighed and incubated in Krebs-Ringer bicarbonate buffer containing 10 μmoles/ml of glycerol, pyruvate or glutamate. The tissues were incubated in a shaking water bath (90 strokes/minute) at 37° and under 95% O_2–5% CO_2. At the end of the 1-hour incubation period the slices were removed, and the glucose content of the medium was assayed essentially as described by Krebs et al. (10) using a glucose oxidase reagent.[3]

For the estimation of gluconeogenesis in vivo two procedures were used. In the initial experiment rats maintained without food for 22 hours were given 500 mg/100 g body weight of glycerol, pyruvate or glutamate. These substrates were administered orally as a 25% solution. Three hours later the animals were killed by decapitation, blood was collected, and liver and diaphragm muscle were taken for glycogen analysis. Serum glucose and tissue glycogen were determined as described earlier (2). In a second experiment, glucose synthesis from pyruvate-2-^{14}C was determined using a procedure similar to that described by Friedman et al. (11). Meal-eating and nibbling rats which were maintained without food for 22 hours, fasted for 22 hours and fed for 2 hours, and nibbling rats which had not been fasted were given intraperitoneally 1 ml of saline containing 1.78 μCi of pyruvate-2-^{14}C and 1 mmole of sodium pyruvate. Thirty minutes later the rats were decapitated, and blood was collected in heparinized and silicon-treated beakers. The blood was deproteinized (12), and the protein-free filtrate was passed over a column containing a mixture of IR 120 (H^+ form) and IR 45 (OH^- form). The column eluates were analyzed for glucose[4] and 1-ml portions were mixed with 10 ml of Bray's solution (13) for assay of radioactivity in a liquid scintillation spectrometer.[5] This procedure removes all charged metabolites from the protein-free filtrate, and only neutral compounds appear in the eluate. The only such compound which might possibly influence the results is glycerol, and only nonsignificant amounts of radioactivity could be found in this metabolite. In fact, about 95% of the radioactivity in the eluate could be isolated as a purified osazone derivative. The percentage of administered pyruvate converted to glucose was estimated by assuming a glucose space (extracellular space) of 30% of body weight (11). The 30-minute time period was used since this period had been shown to yield maximum values (11).

Glucose 6-phosphatase (EC. 3.1.3.9) was estimated in liver homogenates by the method of Nordlie and Arion (14). Liver tissue was homogenized in 0.25 M sucrose with a glass-Teflon homogenizer, and the homogenate was centrifuged at 1,000 × *g* for 20 minutes in a refrigerated centrifuge. The resulting supernatant was used for enzyme assay and protein determination (15). The data were statistically evaluated by means of the *t* test.

[2] Rockland Mouse/Rat Diet (complete), Teklad, Inc., Monmouth, Illinois.

[3] Glucostat, a prepared enzymatic glucose reagent, Worthington Biochemical Corporation, Freehold, New Jersey.

[4] See footnote 3.

[5] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Illinois.

RESULTS

The changes in serum glucose as well as liver and diaphragm glycogen content resulting from the oral administration of various gluconeogenic substrates to meal-fed and nibbling rats are shown in table 1. Serum glucose and liver glycogen levels were higher in the meal-fed control rats as compared with nibbling animals. The three substrates studied produced elevations in blood glucose in both meal-fed and nibbling animals. Glycerol and pyruvate also induced hepatic glycogen storage, whereas glutamate was not very effective in this regard. Muscle glycogen was not greatly influenced by any of the treatments with the possible exception of glycerol administration. The body weight data in table 1 clearly show the lower body weight of meal-fed rats, an observation which is consistent with previous findings (9). The average values shown in table 1 were used to estimate the percentage of administered substrate converted to glucose. These esti-

TABLE 1

Serum glucose and glycogen content of liver and diaphragm of meal-fed and nibbling rats given glycerol, pyruvate or glutamate [1]

Substrate administered [1]	Dietary treatment [2]	Body wt	Serum glucose	Glycogen content	
				Liver	Diaphragm
		g	*mg/100 ml*		*mg/g fresh tissue*
None (H_2O)	ME	254 ± 4 [3]	124 ± 2	10.2 ± 1.1	1.29 ± 0.11
	N	314 ± 5	109 ± 3	3.2 ± 0.5	1.12 ± 0.22
	P [4]	< 0.01	< 0.01	< 0.01	ns
Glycerol	ME	238 ± 7	186 ± 7	45.2 ± 1.0	2.64 ± 0.26
	N	311 ± 10	166 ± 4	41.1 ± 1.6	1.56 ± 0.22
	P	< 0.01	< 0.05	ns	< 0.05
Pyruvate	ME	234 ± 5	179 ± 2	14.4 ± 1.0	1.39 ± 0.27
	N	330 ± 5	172 ± 3	20.1 ± 1.1	0.79 ± 0.08
	P	< 0.01	ns	< 0.01	ns
Glutamate	ME	228 ± 8	145 ± 4	8.8 ± 0.8	1.36 ± 0.25
	N	340 ± 5	149 ± 7	6.4 ± 1.0	1.67 ± 0.06
	P	< 0.01	ns	ns	ns

[1] All animals were maintained without food for 22 hours. They were then force-fed 2 ml/100 g body weight of water or a 25% solution of glycerol, sodium pyruvate or sodium glutamate. The animals were killed 3 hours later.
[2] ME = meal-eating; N = nibbling.
[3] Mean for four rats ± SEM.
[4] Probability of differences between meal-eating and nibbling animals being significant; ns = not significant.

TABLE 2

Estimated conversion of administered glycerol, pyruvate and glutamate to glucose and glycogen by meal-fed and nibbling rats [1]

Substrate	Dietary treatment [2]	Blood glucose [3]	Liver glycogen [4]	Muscle glycogen [5]	Total
		% of administered dose converted to glucose			
Glycerol	ME	3.81	22.05	5.49	31.35
	N	3.50	23.89	1.93	29.32
Pyruvate	ME	4.04	3.16	0.52	7.72
	N	4.62	12.74	0	17.36
Glutamate	ME	2.62	0	0.62	3.24
	N	4.99	4.10	4.91	14.00

[1] Calculated from data presented in table 1.
[2] ME = meal-eating; N = nibbling.
[3] Blood glucose space assumed to be 30% of body weight (11).
[4] Liver weight was assumed to be 2.8% of body weight (2), and 1 mg of glycogen was taken to represent 1.1 mg of glucose.
[5] Muscle mass was assumed to be 39% of body weight (2), and 1 mg of glycogen was taken to represent 1.1 mg of glucose.

mates (table 2) illustrate more clearly the differences shown in table 1. The conversion of the three substrates studied to blood glucose was quite similar and did not appear to be influenced by meal feeding. A marked substrate effect, however, is evident with regard to liver glycogen deposition. The relative order of the substrates, with respect to hepatic glycogen storage, was glycerol > pyruvate > glutamate. Also, these substrates appeared to be more effectively utilized for hepatic glycogen deposition in nibbling rats than in meal-eating animals. Only glycerol promoted any significant storage of muscle glycogen and perhaps glutamate in the nibbling rats. However, as the data in table 1 show, the muscle glycogen changes were small and consequently must be interpreted with caution. Also shown in table 2 is an estimate of the total conversion of the administered substrates to glucose. The same order, with respect to the efficiency of conversion, is seen for liver glycogen storage. Glycerol was utilized for glucose synthesis to about the same extent by meal-fed and nibbling rats, whereas pyruvate and glutamate appeared to be utilized more efficiently by the nibbling animals.

The conversion of these same substrates to glucose by kidney cortex slices was studied with tissue from fasted meal-fed and nibbling rats. The results of this experiment, shown in table 3, suggest that tissue from nibbling rats has a superior gluconeogenic capacity. The production of glucose was generally higher by tissue slices from nibbling rats, although only the difference between tissues incubated with glycerol attained statistical significance. Nonetheless, these data when considered along with the experiment summarized in tables 1 and 2 strongly suggest that the gluconeogenic rate in the meal-fed rat is somewhat lower than that in the animal fed ad libitum.

To estimate more accurately the glucose synthesizing capacity of meal-fed and nibbling animals, an experiment was conducted in which the conversion of pyruvate-2-^{14}C to blood glucose was determined. The results of this experiment are shown in table 4. The gluconeogenic rate was increased more than twofold in the nibbling rat by withholding food for 22 hours. The conversion of pyruvate to glucose was significantly lower in fasted meal-fed than in fasted nibbling rats. Refeeding for 2 hours significantly reduced the rate of gluconeo-

TABLE 3

Glucose synthesis in vitro by kidney cortex slices of meal-fed and nibbling rats [1]

Substrate	Meal-eaters	Nibblers	P [2]
10 μmole/ml buffer	*mμmoles glucose produced/100 mg tissue/hr*		
None	242 ± 38 [3]	260 ± 24	ns
Glycerol	496 ± 5	597 ± 33	< 0.05
Pyruvate	794 ± 87	966 ± 130	ns
Glutamate	508 ± 78	440 ± 22	ns

[1] All animals were maintained without food for 22 hours before killing for tissue removal.
[2] Probability of difference being significant; ns = not significant.
[3] Mean ± SE for four rats.

TABLE 4

Conversion of administered pyruvate-2-^{14}C to glucose by meal-fed and nibbling rats [1]

Dietary status [2]	Meal-eating	Nibbling	P [3]
	% of dose converted to glucose		
Fed	—	7.9 ± 0.7 [4]	—
Fasted	15.5 ± 0.4	20.3 ± 1.3	< 0.01
Refed	2.8 ± 0.2	5.3 ± 0.2	< 0.01

[1] All animals were given intraperitoneally 1 ml of saline containing 1.78 μCi of pyruvate-2-^{14}C and 1 mmole of sodium pyruvate; 30 minutes later the animals were killed and blood collected.
[2] Fed animals were not fasted, fasted animals had been maintained without food for 22 hours; refed rats were allowed access to food for 2 hours following a 22-hour period without food.
[3] Probability of differences being significant; ns = not significant.
[4] Mean for five rats ± SE.

TABLE 5

Hepatic glucose 6-phosphatase activity of meal-fed and nibbling rats [1]

Dietary status [1]	Meal-eating	Nibbling	P [2]
		units/mg protein [3]	
Fed	—	98 ± 3(8) [4]	—
Fasted	98 ± 3(10)	114 ± 7(8)	ns
Refed	78 ± 4(10)	81 ± 4(9)	ns

[1] Fed animals had access to food until the time of killing; fasted rats were maintained without food for 22 hours prior to killing; refed animals were maintained without food for 22 hours and were then given access to food for 2 hours. They were killed 6 hours after the start of feeding.
[2] Probability of difference between meal-fed and nibbling animals being significant; ns = not significant.
[3] A unit is described as the liberation of 1 mμmole of phosphate per minute at 30°.
[4] Mean ± SE for number of rats indicated in parentheses.

genesis in both groups but the meal-fed rats still converted significantly less pyruvate to glucose than did the nibbling animals.

Data are presented in table 5 on the hepatic glucose 6-phosphatase activity of meal-fed and nibbling rats. The activity of this enzyme was similar for meal-fed and nibbling rats and decreased significantly in both groups upon refeeding.

DISCUSSION

The previously reported increase in cytoplasmic pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities in liver of meal-fed rats (6) suggested that meal feeding might influence the gluconeogenic capacity of rats. The results presented in this report, however, clearly show that the gluconeogenic rate of fasted meal-fed rats is not enhanced over that of fasted nibblers but, in fact, is probably lower.

These data also suggest that the meal-fed animal is better able to suppress glucose synthesis upon meal ingestion (table 4). In animals maintained without food for 22 hours and allowed to eat for 2 hours, the conversion of pyruvate-2-^{14}C to glucose by the meal-fed rat was only about half of that by the nibbling animals. This depression in glucose synthesis apparently can be related to the reduction in circulating free fatty acids which follows food ingestion (4, 16–20). This effect appears to be brought about by increased glucose availability and circulating insulin levels induced by food ingestion. Under these conditions entry of glucose into adipose tissue cells is enhanced, and the synthesis of α-glycerophosphate increases, thereby stimulating triglyceride formation and decreasing free fatty acid release. In liver, fatty acids appear to inhibit several glycolytic steps and tend to stimulate a reversal of glycolysis and, hence, gluconeogenesis (18, 19). A reduction in circulating free fatty acids would overcome their inhibitory effects on glycolysis, thereby enhancing glucose utilization and suppressing gluconeogenesis.

The present experiments suggest that gluconeogenesis is more effectively suppressed by food ingestion following a fast in meal-fed than in nibbling rats. Although the mechanism involved is unknown, the possible role of insulin provides an attractive hypothesis. The meal-fed rat has been shown to absorb glucose at a rate 40% greater than that of the nibbling animal; yet blood glucose increases less rapidly in the meal-fed rat (21). Consequently, glucose uptake by tissues of the meal-fed animal must be more rapid and may be related to an enhanced sensitivity of tissues to insulin or perhaps a greater release of the hormone. In either case, a more rapid uptake of glucose by adipose tissue could result in a depression of fatty acid release and, hence, of gluconeogenesis by the sequence of events outlined above.

The lowered rate of gluconeogenesis observed in fasted meal-fed rats suggests that as a consequence of meal eating the animal develops the ability to utilize glucose more judiciously during periods of fast. It might be expected for example, that the utilization of glucose and its turnover rate would be lower in the fasted meal-fed animal than in its nibbling counterpart. The experiments reported, when viewed together, suggest that the major effect of meal feeding on gluconeogenesis in the rat is largely one of increasing the sensitivity of the regulatory mechanisms controlling glucose formation.

The diurnal variations in hepatic glucose 6-phosphatase activity reported by Freedland and Harper (5) for meal-fed rats has been confirmed by the present investigations. As the present data show, however, the changes are not unique for the meal-fed rat since similar variations were observed in fasted and fasted-refed nibbling animals (table 5). Furthermore, no significant difference was observed between the hepatic enzyme activities of meal-fed and nibbling rats.

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Effect of Dietary Magnesium on Strontium-Calcium Discrimination and Incorporation into Bone of Rats [1]

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ABSTRACT Although strontium-calcium discrimination from diet to body (OR (observed ratio) body/diet) is relatively constant under typical conditions, it can be changed by dietary modifications. Since magnesium is known to affect calcium absorption, a study was made in rats of its effect on calcium and strontium absorption and on strontium-calcium discrimination. The OR bone/diet was markedly increased at dietary magnesium levels below about 0.4%. From a practical standpoint it is noted that the body burden of ^{90}Sr from chronic ingestion decreases in direct proportion to the decrease caused in the value of OR body/diet, divided by the percentage dietary calcium (OR/% Ca). Previous attempts to reduce this value to a minimum by manipulation of calcium and phosphorus levels gave an OR/% Ca of about 0.1. In this study, by use of a diet containing 4% Ca, 4% P, and 0.5% Mg, a further reduction to 0.06 was obtained. Also, there is suggested a possible explanation for the varying results reported in the literature on the effect of dietary magnesium on calcium absorption.

Supplemental dietary magnesium has been reported to increase calcium absorption (1–4), to decrease calcium absorption (5–8), or to have no effect on calcium absorption (9–13). The existence of a common mechanism for the intestinal absorption of calcium and magnesium has been postulated (14–16), and relationships with phosphorus have been demonstrated (17–18). Since strontium-calcium absorption and retention are known to be related to phosphorus levels, the present study was undertaken to determine the effect of a wide range of dietary magnesium levels on the retention of ^{47}Ca and ^{85}Sr in the femurs of growing rats at relatively high calcium and phosphorus dietary levels. Dietary calcium and phosphorus levels of 2 to 4% were used because it has been shown (19) that they tend to reduce the relative body burden of ingested strontium that will be developed, and it was of particular interest to determine if modification of magnesium levels would increase the effect.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain, initially weighing 150 g, were used for all diet experiments. When received, rats were randomly distributed into individual wire-mesh cages and allowed to adjust to their environment for a period of 2 days, while being fed a commercial diet [2] and deionized water ad libitum. On day 1 of each experiment the rats were given their respective experimental diets (table 1) and allowed free access to deionized water. The diets were continued throughout the experimental period with feed and water consumption recorded daily. Doses of ^{85}Sr and ^{47}Ca were administered on days 9 and 10. Each dose consisted of 0.5 μCi of ^{85}Sr and 1.0 μCi of ^{47}Ca (specific activity 0.18 Ci/g Ca) in 50 ml of deionized water. This was given in graduated glass drinking tubes in 25-ml daily portions. Twenty-four hours after dosing was completed, the rats were killed by ether anesthesia and cardiac injection of sodium pentobarbital. Both femurs of each rat were removed, cleaned and placed in counting vials. Samples were then counted simultaneously for ^{85}Sr and ^{47}Ca content with a gamma spectrometer using a sodium iodide crystal. Pulse height analysis was used to reduce background and to discriminate between gamma emissions of the radionuclides. Experimental

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[2] Big-Red Rat Diet, Agway, Incorporated, Syracuse, New York.

TABLE 1

Diet composition for dietary magnesium experiments

Component	Exp. A, B, C	Exp. D	Exp. E
	%	%	%
Sucrose	53.0	52.0	51.0
Vitamin-free casein [1]	18.0	18.0	18.0
Vegetable oil [2]	3.0	3.0	3.0
Vitamin supplement [3]	2.2	2.2	2.2
Salt mix [4]	6.0	6.0	6.0
$Ca(H_2PO_4)_2 \cdot H_2O$	8.4	11.5	15.7
$CaCO_3$	—	1.3	2.1
$MgCO_3$ and Alphacel [5]	9.4	6.0	2.0
	100.0	100.0	100.0
Mineral content			
Calcium	2.0	3.0	4.0
Phosphorus	2.3	3.0	4.0
Magnesium [6]		See table 2	

[1] Vitamin Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[2] Wesson Oil, Wesson Sales Company, Fullerton, California.

[3] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, containing per 45.4 kg diet: (in grams) vitamin A concentrate, 200,000 IU/g, 4.5; vitamin D concentrate, 400,000 IU/g, 0.25; α-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; thiamine·HCl, 1.0; calcium pantothenate, 3.0; (in milligrams) biotin, 20.0; folic acid, 90.0; and vitamin B_{12}, 1.35.

[4] Magnesium-free salt mix, Nutritional Biochemicals Corporation, containing per 45.4 kg diet: (in grams) calcium carbonate, 906.0; monocalcium phosphate, 227.0; copper sulfate, 920.0; manganous sulfate, 12.0; ferric citrate, 84.5; potassium iodide, 2.5; sodium chloride, 507.0; dipotassium phosphate, 227.0; and (in milligrams) zinc carbonate, 760.0.

[5] Alphacel - Hydrolyzed, Nutritional Biochemicals Corporation.

[6] Basal diet contained 20 ppm magnesium. Values for percent dietary magnesium in the various experimental diets in tables 2 and 3 represent the amount of magnesium added as $MgCO_3$.

results were expressed as $OR_{bone/diet}$ [3] and percent dose of ^{47}Ca and ^{85}Sr in the femur.

RESULTS

The data on feed consumption and weight gain are presented in table 2. It is noted that there was no increase in body weight at dietary levels of 0.1% magnesium or lower; there was some growth at 0.2% magnesium, but 0.4 to 0.5% was required for maximum growth. Since all the calcium and phosphorus levels were high, no important effects on growth were expected or seen. However, there were some indications: at a low level of magnesium (0.05%), increasing the calcium and phosphorus from 2 to 4% decreased feed intake and growth; at the optimum level of magnesium (0.5%), the best response was obtained with 3% each of calcium and phosphorus as compared with 2% and 4%, respectively; at a high level of magnesium (1.5%), increasing the calcium and phosphorus from 2 to 3% increased feed intake and growth. Because of the nature of the study, statistical analysis was possible only within experimental groups. A summary of the statistics is presented in table 3. In terms of increase in body weight the following is noted: In experiment A there was a significant increase between the basal diet and the 0.5% added magnesium; in experiment B, between 0.05% and 0.5% added magnesium; in experiment E, between 0.1 ond 0.2%, and between 0.3 and 0.4% added magnesium.

Table 4 presents data of the effects of dietary magnesium at three levels of calcium and phosphorus on the deposition and retention of ^{47}Ca and ^{85}Sr in bone and on values of $OR_{bone/diet}$. It shows that an increase in dietary magnesium at low levels (0 to 0.5%) tended to decrease the amounts of both ^{47}Ca and ^{85}Sr in the femur; this is opposite to the effect at high magnesium levels (0.5 to 2.5%) where an increase in dietary magnesium tended to increase the amounts of ^{47}Ca and ^{85}Sr in the femur. The effect of changing the dietary level of calcium and phosphorus was not as clear. There were five groups among which comparisons could be made: For ^{47}Ca there were no effects except for one comparison in which changing from 2 to 3% calcium and phosphorus increased the amount of ^{47}Ca in bone. In contrast, every increase in calcium and phosphorus was reflected by an increased amount of ^{85}Sr found in bone.

The comparative behavior of strontium and calcium can best be assessed by consideration of the values of $OR_{bone/diet}$. It appears clear that increasing the added dietary levels of magnesium from zero to about 0.3% decreased the OR values; at higher dietary levels of magnesium there were no further effects. Increasing the dietary levels of calcium and phosphorus from 2 to 4% caused an increase in the OR values. This is in general agreement with findings of Kostial et al. (19).

[3] Observed ratios, $OR_{sample/precursor} = \frac{\text{Sr/Ca of sample}}{\text{Sr/Ca of precursor}}$ describes the comparative movement of strontium and calcium into tissues or excretions.

TABLE 2

Experimental design, feed consumption, and weight changes in rats

Exp.	No. of rats	Added magnesium [1]	Ca	P	Feed intake	Body wt change
		%	%	%	*g/rat/11 days*	*g*
A	6	0	2.0	2.3	66	−36
B	6	0.0005	2.0	2.3	81	−24
B	5	0.005	2.0	2.3	90	−16
B	6	0.05	2.0	2.3	117	−10
E	7	0.05	4.0	4.0	91	−27
E	7	0.1	4.0	4.0	115	−13
E	7	0.2	4.0	4.0	143	+19
E	7	0.3	4.0	4.0	141	+15
E	7	0.4	4.0	4.0	160	+32
E	7	0.5	4.0	4.0	171	+37
D	10	0.5	3.0	3.0	195	+48
C	6	0.5	2.0	2.3	66	−32
C	6	0.5	2.0	2.3	88	−14
C	6	0.5	2.0	2.3	110	− 4
C	6	0.5	2.0	2.3	132	+ 6
C	6	0.5	2.0	2.3	145	+15
B	6	0.5	2.0	2.3	163	+32
A	7	0.5	2.0	2.3	151	+27
A	7	1.0	2.0	2.3	163	+30
A	7	1.5	2.0	2.3	169	+28
D	10	1.5	3.0	3.0	204	+55
A	7	2.0	2.0	2.3	142	+24
A	7	2.5	2.0	2.3	146	+27

[1] Basal diet contained 20 ppm Mg.

TABLE 3

Statistical analyses of experimental means [1]

Exp.	Added magnesium [2]	Feed intake [3]	Body wt change [3]	^{47}Ca in femur	^{85}Sr in femur	$OR_{bone/diet}$
	%	*g/rat/11 days*	*g*	*% dose*	*% dose*	
A	0	66 ± 8 [a]	−36 ± 4 [a]	1.2 [a]	0.50 [a,b]	0.42 [a]
	0.5	151 ± 8 [b]	+27 ± 4 [b]	1.7 [a]	0.31 [c,d]	0.18 [b]
	1.0	163 ± 8 [b]	+30 ± 4 [b]	1.5 [a]	0.26 [d]	0.18 [b]
	1.5	169 ± 8 [b]	+28 ± 4 [b]	1.7 [a]	0.35 [c,d]	0.20 [b]
	2.0	142 ± 8 [b]	+24 ± 4 [b]	2.2 [b]	0.44 [b,c]	0.20 [b]
	2.5	146 ± 8 [b]	+27 ± 4 [b]	2.8 [c]	0.60 [a]	0.21 [b]
B	0.0005	81 ± 4 [a]	−24 ± 4 [a]	1.0 [a]	0.43 [a]	0.40 [a]
	0.005	90 ± 4 [a]	−16 ± 4 [a]	0.9 [a]	0.34 [a,b]	0.36 [a,b]
	0.05	117 ± 4 [b]	−10 ± 4 [a]	0.9 [a]	0.29 [b]	0.32 [b]
	0.5	163 ± 4 [c]	+32 ± 4 [b]	1.6 [b]	0.28 [b]	0.18 [c]
C	0.5	66 6 g/day [4]	−32 ± 2 [a]	1.4 [a]	0.32 [a]	0.23 [a]
	0.5	88 8 g/day	−14 ± 2 [b]	1.3 [a]	0.30 [a]	0.23 [a]
	0.5	110 10 g/day	− 4 ± 2 [c]	1.0 [a]	0.21 [b]	0.20 [a]
	0.5	132 12 g/day	+ 6 ± 2 [d]	1.1 [a]	0.19 [b]	0.18 [a]
	0.5	145 ad lib	+15 ± 2 [e]	1.1 [a]	0.20 [b]	0.18 [a]
D	0.5	195 ± 5 [a]	+48 ± 2 [a]	1.7 [a]	0.34 [a]	0.20 [a]
	1.5	204 ± 5 [a]	+55 ± 2 [a]	2.5 [b]	0.51 [b]	0.20 [a]
E	0.05	91 ± 6 [a]	−27 ± 5 [a]	0.9 [a]	0.38 [a]	0.45 [a]
	0.1	115 ± 6 [b]	−13 ± 5 [a]	0.8 [a]	0.29 [a]	0.38 [b]
	0.2	143 ± 6 [c]	+19 ± 5 [b]	1.3 [b]	0.37 [a]	0.29 [c]
	0.3	141 ± 6 [c]	+15 ± 5 [b]	1.3 [b]	0.35 [a]	0.26 [c,d]
	0.4	160 ± 6 [c,d]	+32 ± 5 [c]	1.3 [b]	0.33 [a]	0.25 [d]
	0.5	171 ± 6 [d]	+37 ± 5 [c]	1.5 [b]	0.38 [a]	0.25 [d]

[1] Duncan's new multiple range test. Any two means not followed by the same letter are significantly different ($P < 0.05$). Any two means which are followed by the same letter are not significantly different.

[2] Basal diet contained 20 ppm magnesium.

[3] Group mean ± SEM.

[4] Controlled daily feed consumption.

TABLE 4

Effect of dietary magnesium levels on ^{47}Ca and ^{85}Sr deposition and retention in bone and on values of $OR_{bone/diet}$[1]

Dietary Ca and P, %	^{47}Ca in femur, % of dose			^{85}Sr in femur, % of dose			$OR_{bone/diet}$		
	2	3	4	2	3	4	2	3	4
% added magnesium [2]									
0	1.2 ± 0.1			0.50 ± 0.04			0.42 ± 0.01		
0.0005	1.0 ± 0.1			0.43 ± 0.04			0.40 ± 0.01		
0.005	0.9 ± 0.1			0.34 ± 0.04			0.36 ± 0.01		
0.05	0.9 ± 0.1		0.9 ± 0.1	0.29 ± 0.04		0.38 ± 0.03	0.32 ± 0.01		0.45 ± 0.01
0.1			0.8 ± 0.1			0.29 ± 0.03			0.38 ± 0.01
0.2			1.3 ± 0.1			0.37 ± 0.03			0.29 ± 0.01
0.3			1.3 ± 0.1			0.35 ± 0.03			0.26 ± 0.01
0.4			1.3 ± 0.1			0.33 ± 0.03			0.25 ± 0.01
0.5	1.6 ± 0.1 [3]	1.7 ± 0.1	1.5 ± 0.1	0.30 ± 0.04 [3]	0.34 ± 0.03	0.38 ± 0.03	0.18 ± 0.01 [3]	0.20 ± 0.01	0.25 ± 0.01
1.0	1.5 ± 0.1			0.26 ± 0.04			0.18 ± 0.01		
1.5	1.7 ± 0.1	2.5 ± 0.1		0.35 ± 0.04	0.51 ± 0.03		0.20 ± 0.01	0.20 ± 0.01	
2.0	2.2 ± 0.1			0.44 ± 0.04			0.20 ± 0.01		
2.5	2.8 ± 0.1			0.60 ± 0.04			0.21 ± 0.01		

[1] Observed ratios, $OR_{sample/precursor} = \frac{Sr/Ca \text{ of sample}}{Sr/Ca \text{ of precursor}}$. All values are group means ± SEM.

[2] Basal diet contained 20 ppm magnesium.

[3] Average of means from experiments A and B (see table 3).

Since the most marked changes in OR values occurred under conditions of suboptimal feed intake and growth, a further experiment was done to see if these two factors alone had any effect on the OR value. Experiment C was carried out exactly as the other studies, with a constant dietary level of magnesium at 0.5%, but with feed intake restricted to 66, 88, 110, 132 and 145 (ad libitum) g/rat/11 days; this resulted in the following respective weight changes: − 32, − 14, − 4, + 6 and + 15 g. The corresponding OR values were: 0.23, 0.23, 0.20, 0.18 and 0.18. Thus, restricted feed intake and the resultant weight loss did not contribute importantly to the changes noted in the OR values.

DISCUSSION

In this study, levels of dietary magnesium of about 0.4% were required for optimum growth response. This is much higher, for example, than the levels of 0.064% recently reported to be adequate when the calcium and phosphorus contents were about 1% (20). These results demonstrate again that deficiencies and response to magnesium must take into account at least the concurrent levels of calcium and phosphorus.

The variable results that have appeared in the literature on the effect of magnesium level on the retention of calcium can be explained by reference to figure 1. This schematic illustration was prepared from the data obtained in the present study and is proposed to characterize the effect of increasing dietary magnesium on calcium retention; numerical values are not given since they will vary with the particular levels of calcium, phosphorus and magnesium utilized. It is pointed out that according to this representation a given increase in dietary magnesium level could cause: a) a relatively small decrease in calcium retention, b) a sharp increase, c) no change, or d) a relatively small increase, all depending upon the region of the curve in which the changes were being made.

In regard to the comparative behavior of calcium and strontium, it has been noted that increasing the magnesium level tended to decrease the OR value, that is, to increase the rat's discrimination against strontium relative to calcium. It is noted

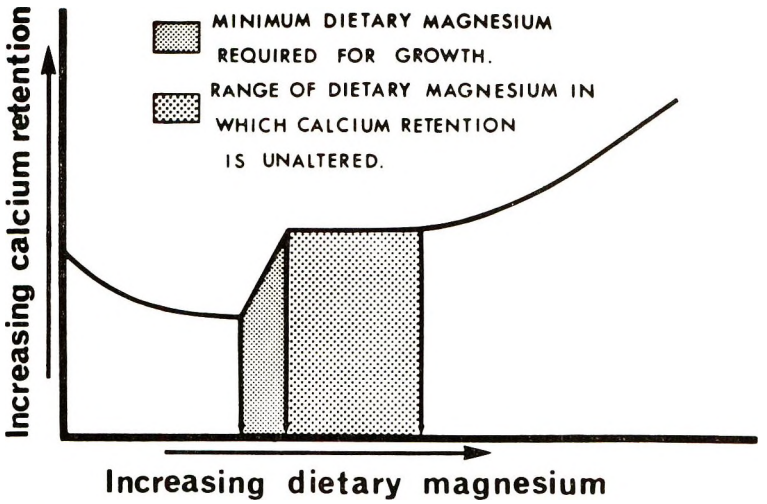

Fig. 1 Stylized curve for relationship of dietary magnesium and calcium retention.

from table 4 that this was brought about by the fact that increased dietary magnesium either decreased ^{47}Ca retention to a lesser degree than ^{85}Sr retention, or else increased ^{47}Ca retention proportionally more than ^{85}Sr retention.

From a practical standpoint there is an interest in being able to manipulate diets so that the body burden of radioactive strontium resulting from a given diet will be at a minimum. As pointed out by Kostial et al. (19), the lower the value of $OR_{body/diet} \div$ % Ca in a diet (OR/% Ca), the lower will be the developed body burden from a given level of dietary radioactive strontium. These workers were able to obtain a minimum value of OR/% Ca of about 0.1 at levels of calcium and phosphorus at 3%, and with no information available on the magnesium content of the diet; this represented about a fivefold decrease in the OR/% Ca from that observed at levels of 0.5% Ca and P. As can be calculated from table 4, a minimum OR/% Ca value of about 0.06 was obtained at 4% Ca, 4% P and 0.4–0.5% Mg. From a practical standpoint if diets are to be modified to produce low body burdens, the procedure suggested by these experiments would be to increase to a maximum the dietary levels of phosphorus and uncontaminated calcium and to increase dietary magnesium sufficiently both to effect additional strontium-calcium discrimination and to provide for an increased magnesium requirement.

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Effect of Sorbitol, Fructose, Succinate, Aspartate, Glutamate and Fat on Growth and Survival Time of Biotin-deficient Rats [1,2]

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ABSTRACT In a series of experiments (randomized complete block design) using weanling male rats, the effect of sorbitol, fructose, sodium succinate, aspartate and glutamate at a level of 5 or 10% (at the expense of glucose in the basal diet containing 20% spray-dried egg white) on growth and survival time of biotin-deficient rats was studied. The test substance was included at the beginning of the experiment or after 4 weeks on the basal diet. Under these conditions no significant effect on growth or survival time of biotin-deficient rats was observed. Addition of 10 or 20% fat to the basal diet containing 4% corn oil resulted in a significant increase in growth and survival time of deficient animals.

In contrast with the observations of Herndon et al.[3] Morgan and Yudkin (1) reported a biotin-sparing effect of sorbitol on body weights of rats fed a diet containing avidin. The restoration of protein synthesis to normal levels in biotin-deficient rats fed the spray-dried egg white diet containing sorbitol, frutose or succinate has been reported by Mistry and co-workers (2–5). The biotin-sparing effect of aspartate and fatty acids on growth of lactic acid bacteria (6) and yeast (7) grown in media containing suboptimal amounts of biotin has been observed.

Although the beneficial effects of sorbitol, fructose or succinate on protein synthesis in biotin-deficient rats have been reported, the growth-promoting effect of these compounds has remained controversial or has not been studied. Therefore, we have reinvestigated the effect of sorbitol and fructose on growth and survival time of biotin-deficient rats. We have also studied the effect of sodium succinate, aspartate, glutamate and fat on growth and survival time of these animals.

MATERIALS AND METHODS

Weanling male rats of the Sprague-Dawley strain were housed individually in metal cages with raised wire screens in a temperature-regulated room. The composition of the basal diet is given in table 1. Other diets, with the exception of rations containing fat, were prepared by adding 5 or 10% of the test substance to the basal diet at the expense of an equivalent amount of glucose. In diets containing 10 or 20% fat,[4] the level of protein was varied to maintain a constant calorie-to-protein ratio. Since the basal diet already contained 4% corn oil, the actual fat content of these two diets was 14 and 24%, respectively. All animals were fed ad libitum, and water was available at all times.

In a series of experiments the effect of fructose, sorbitol, glutamate, aspartate and fat was investigated during various stages of the deficiency. In these studies, the animals were fed the basal diet for the first 4 to 7 weeks of the experiment (table 2). At this time the deficient animals were grouped so that each group had approximately the same mean body weight and a similar weight distribution (randomized complete block design (RCB design)). The various diets were assigned randomly to these groups, and the animals were con-

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[2] Preliminary report was made at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, 1968 Federation Proc., 27: 554 (abstract).

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[4] Crisco, Procter and Gamble, Cincinnati.

TABLE 1

Composition of the basal diet

	%
Glucose [1]	70.64
Spray-dried egg white [2]	20.00
Salt 4164 [3]	4.00
Corn oil [4]	4.00
Vitamin B mix [5]	1.00
Vitamin A and D mix [6]	0.25
Choline chloride	0.10
Vitamin E acetate [7]	0.01

[1] Cerelose, Corn Products Company, New York.

[2] Nutritional Biochemicals Corporation, Cleveland.

[3] Salt mixture no. 4164, General Biochemicals Incorporated, Chagrin Falls, Ohio (18).

[4] Mazola (pure corn oil), Corn Products Company, New York.

[5] Composition of vitamin B mix: (percentage of mix) ascorbic acid, 2.5; thiamine·HCl, 1.0; niacin, 1.0; i-inositol (meso), 1.0; Ca-D-pantothenate, 0.4; riboflavin, 0.15; pyridoxine·HCl, 0.06; menadione, 0.05; folic acid, 0.04; p-aminobenzoic acid, 0.02; vitamin B_{12} (0.1% trituration with mannitol), 0.20; and glucose, 93.58.

[6] Composition of vitamin A and D (both water dispersible): Vitamin A palmitate, 1.2×10^6 IU; vitamin D_2, 1.2×10^5 IU; and glucose to make up to 150 g; 100 g of diet contained vitamin A palmitate, 2000 IU; and vitamin D_2, 200 IU.

[7] *dl*-α-Tocopheryl acetate, General Biochemicals Incorporated, Chagrin Falls, Ohio. Vitamin E was premixed with corn oil before it was added to the diet.

tinued on their diets till they died of biotin deficiency.

In another series of experiments the effect of these dietary ingredients and also sodium succinate was tested from the beginning of the experiment. Thirty-two to 38-g rats were divided into groups of 10 or 15 rats with approximately the same mean body weight and a similar weight distribution in each group (RCB design). The animals were assigned the various diets at random, and they were continued on these regimens till they died of biotin deficiency. Body weights were recorded at about weekly intervals. In one experiment, food intake was also recorded. The survival time is defined as the number of days the animal survived on the experiment.

In the study on the effect of fructose and sorbitol on the cecum and large intestine of biotin-deficient rats, the animals were decapitated and exsanguinated; tissues were removed and weighed with their contents. The cecum and large intestine

TABLE 2

Effect of fructose, sorbitol, glutamate, aspartate and fat on survival time of biotin-deficient rats during the later stage of the deficiency

Exp. no.	Initial body wt range	Initial period fed basal diet	Treatment		Survival time	P value [4]
			Supplement to basal diet [1]	Body wt, start of treatment [2]		
	g	*weeks*	%	*g*	*days*	
1	37–45	7	none	145 ± 4(9) [3]	79 ± 3	
			10 fructose	144 ± 5(9)	73 ± 2	ns
			5 glutamate	146 ± 4(9)	83 ± 2	ns
2	31–38	6	none	110 ± 5(9)	60 ± 3	
			10 fructose	109 ± 5(9)	56 ± 2	ns
			5 glutamate	110 ± 5(9)	64 ± 2	ns
3	31–38	4	none	115 ± 2(10)	53 ± 1	
			10 fructose	114 ± 2(10)	53 ± 3	ns
			10 sorbitol	114 ± 2(10)	53 ± 2	ns
4	31–38	4	none	112 ± 4(8)	54 ± 2	
			5 aspartate	114 ± 3(7)	53 ± 2	ns
			10 fat	111 ± 4(8)	69 ± 3	< 0.001
5	31–38	4	none	106 ± 3(10)	52 ± 2	
			5 aspartate	110 ± 4(10)	56 ± 3	ns
			10 fat	106 ± 4(10)	59 ± 2	< 0.01

[1] The supplement was added at the expense of an equal amount of glucose to the basal diet. Upon adding fat at a level of 10 or 20% to the basal diet containing 4% corn oil the level of protein in the diet was varied to maintain a constant calorie-to-protein ratio.

[2] Randomized complete block design at the start of the treatment for all experiments except experiment 5 where the animals were grouped at the beginning of the experiment.

[3] Each result is the mean ± SE of the mean. The numbers of animals are shown in parentheses.

[4] ns = nonsignificant.

were then flushed with water, blotted, weighed on a precision balance, and dried to constant weight at 60°.

RESULTS

The effect of sorbitol, fructose, aspartate, glutamate and fat on the survival time of biotin-deficient rats during various stages of the deficiency is shown in table 2. With the exception of fat none of these compounds had a beneficial effect. Also, aspartate or glutamate had no effect on gain in body weight (fig. 1 A), food intake or survival time (table 3) of biotin-deficient animals, even when fed from the beginning of the experiment. Although addition of 5 or 10% sodium succinate led to a slight decrease in weight gain (fig. 1 A), it had no effect on survival time of the deficient animals (table 3).

The results of a study on growth and survival time of biotin-deficient rats fed fructose or sorbitol at a level of 10% from the start of the experiment are given in figure 1B and table 3. As before, the sugars had no effect on survival time. Also, there was no difference in the growth rate of these animals up to about 3 weeks. After this period the growth rate of animals fed sorbitol appeared to increase, compared with animals fed the basal or the fructose diet, and reached a peak at about 5 weeks. Table 4 shows the effect of these sugars on the cecum and large intestine of biotin-deficient rats. These tissues were significantly heavier in animals fed sorbitol compared with rats fed the basal or the fructose diet.

The effect of feeding fat on weight gain and survival time of biotin-deficient rats is seen in figure 1 C and table 3. The deficient animals fed the diets containing 10 or 20% fat gained significantly more weight and survived a significantly longer time than animals fed the basal diet. An increase in weight gain of biotin-deficient rats fed diets containing zero, 2 and 20% corn oil has been reported (8).

DISCUSSION

Morgan and Yudkin (1), in contrast to the observations of Herndon et al.,[5] reported a biotin-sparing effect of sorbitol on body weights of deficient rats. Addition of sorbitol or fructose to the basal diet was reported to restore amino acid incorporation to normal levels in microsomal proteins of biotin-deficient rat livers (4,5,9). It was suggested that compared with glucose these sugars were better utilized by the deficient animal for energy production.

[5] See footnote 3.

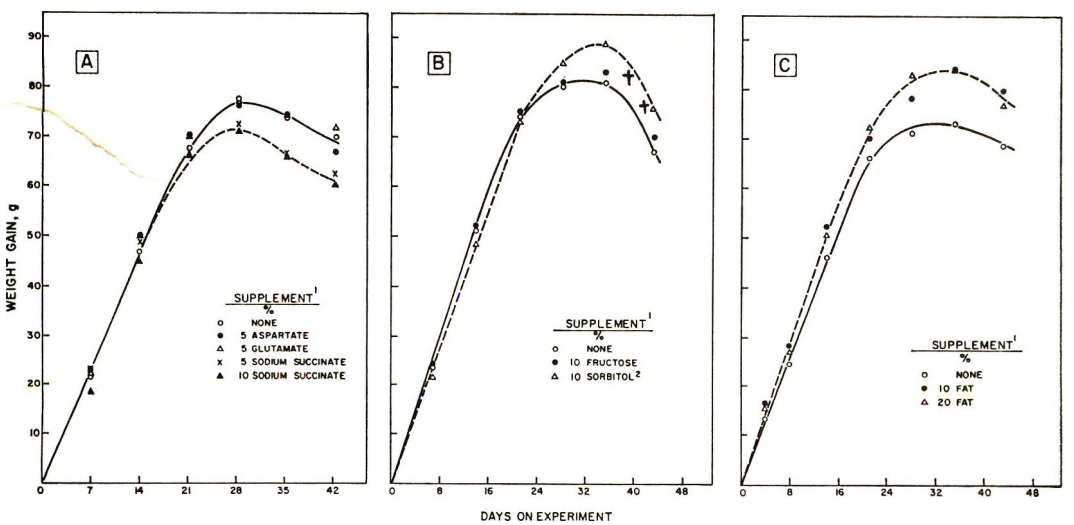

Fig. 1 Effect of aspartate, glutamate, sodium succinate, fructose, sorbitol and fat on weight gain of biotin-deficient rats. The mean body weight in each group at the start of the experiment (randomized complete block design) was 34 g. The animals were fed ad libitum the assigned diets from the start of the experiment. Each result is the mean of 10 rats. [1] See footnote 1 in table 2. [2] See footnote 3 in table 3. † Indicates the death of an animal in the group fed sorbitol.

TABLE 3

Effect of aspartate, glutamate, sodium succinate, fructose, sorbitol and fat on survival time of biotin-deficient rats

Exp. no.	Supplement to basal diet [1]	Food intake [2]	Survival time	P value [3]
	%	*g/rat/day*	*days*	
	none		52 ± 1	
	5 aspartate		54 ± 2	ns
1	5 glutamate		53 ± 1	ns
	5 sodium succinate		51 ± 2	ns
	10 sodium succinate		51 ± 2	ns
	none	8.5	54 ± 1	
2	5 aspartate	8.3	54 ± 1	ns
	5 glutamate	8.8	55 ± 2	ns
	none		52 ± 1	
3	5 glutamate		55 ± 2	ns
	10 egg-white, spray-dried		52 ± 2	ns
	none		50 ± 2	
4	10 fructose		52 ± 2	ns
	10 sorbitol [4]		48 ± 1	ns
	none		52 ± 1	
5	10 fat		61 ± 3	< 0.01
	20 fat		59 ± 2	< 0.01

The mean body weight in each group at the start of the experiment (randomized complete block design) was 34 g. The animals were fed ad libitum the assigned diets from the start of the experiment. Each result is the mean ± SE of the mean of 10 rats for all experiments except experiment 2 where 15 rats were used for each group.

[1] See footnote 1 in table 2.

[2] Food intake was recorded only for 6 days (days 22 to 27) in experiment 2.

[3] ns = nonsignificant.

[4] For the first 10 days of the experiment sorbitol was substituted for glucose only at a level of 5%.

In the present study we found no effect of sorbitol or fructose on growth or survival time of biotin-deficient rats. The apparent increase in body weights of biotin-deficient animals fed the sorbitol diet could be accounted for partly by the increase in the weight of cecum and large intestine and their contents (table 4). The same effect of sorbitol on these tissues was observed by Morgan and Yudkin (10) in thiamine-deficient rats.

In earlier studies we demonstrated a beneficial effect of feeding succinate to biotin-deficient rats and chickens on protein synthesis (2,3) and suggested that the exogenous addition of succinate overcame the impairment of dicarboxylic acid synthesis. Although succinate did have a beneficial effect on protein synthesis in biotin deficiency, succinate, aspartate and glutamate at the levels tested did not have a beneficial effect on growth (fig. 1A), food intake or survival time of rats during various stages of the deficiency (tables 2 and 3). The slight depression in growth observed in animals receiving 5 or 10% sodium succinate was probably due to excess sodium in these diets. Twenty percent egg white in the basal diet supplied about 5% aspartate and glutamate (11) in the basal diet. The absence of any effect of additional aspartate or glutamate on growth was not due to a dilution of the essential amino acids, since increasing the level of egg white in the diet from 20 to 30% also had no effect on survival time of biotin-deficient rats (table 3).

Acetyl-CoA carboxylase, a biotin-containing enzyme, has been shown to be inhibited when the animal is fed a diet rich in fat (12). The level of acetyl-CoA carboxylase in the livers of rats and chickens was reduced in biotin deficiency (13) although even in severe deficiency the enzyme was never completely absent (14, 15). Liver mitochondria of biotin-deficient rats contained markedly less fatty acids, particularly palmitate and stearate. Also, the incorporation of acetate-1-^{14}C into mitochondrial fatty acids of rats was reduced in the deficiency (16). The gradual decrease in the lipid content of liver mito-

TABLE 4

Effect of fructose and sorbitol on cecum and large intestine of biotin-deficient rats

Supplement [1]	Body wt	Wet wt				Dry wt		Wet wt with content
		With content		Without content				
		CM [3]	LI [4]	CM	LI	CM	LI	CM and LI
%	*g*	*g*	*g*	*g*	*g*	*mg*	*mg*	*g*
None	103 ± 4(10)	2.16 ± 0.14	0.71 ± 0.05	0.30 ± 0.01	0.36 ± 0.02	45 ± 3	67 ± 3	2.9
10 fructose	101 ± 2(10)	1.88 ± 0.20	0.65 ± 0.05	0.32 ± 0.02	0.41 ± 0.01	48 ± 3	67 ± 3	2.5
10 sorbitol [2]	105 ± 2(6)	4.15 ± 0.26	1.46 ± 0.14	1.23 ± 0.09	1.11 ± 0.06	193 ± 17	178 ± 8	5.6

The animals were fed ad libitum the assigned diets from the start of the experiment. Each result is the mean ± SE of the mean. The numbers of animals are shown in parentheses.

[1] See footnote 1 in table 2.

[2] See footnote 3 in table 3.

[3] Cecum.

[4] Large intestine.

chondria observed with the progress of biotin deficiency correlated with the progressive decrease in oxidative phosphorylation (4,5). A relatively reduced incorporation of fatty acids into triglycerides, compared with the incorporation into phospholipids, has been shown in biotin-deficient chicks (17). The respiratory quotient (0.85) of rats fed ad libitum a diet high in carbohydrate and low in fat indicates that animals derive about 50% of their energy from the oxidation of fatty acids (18). The basal diet used in the present study is also high in carbohydrate and low in fat. In view of the very marked reduction in fatty acid synthesis in biotin deficiency, the availability of fatty acids for oxidation in deficient animals is markedly reduced. Therefore, the beneficial effect of adding 10 or 20% fat to the basal diet containing 4% corn oil on rate of growth (fig. 1C) and survival time (tables 2 and 3) of biotin-deficient rats could be due to a) reduced lipogenesis as a result of feedback inhibition by fatty acids supplied in the diet, resulting in a decreased depletion of initial stores of biotin in the animals; b) increased incorporation of dietary fatty acids into structurally important phospholipids such as mitochondrial lipids, thereby helping to maintain the integrity of mitochondria and improved oxidative phosphorylation; and c) the utilization of dietary fatty acids for the production of energy, since glucose metabolism is impaired in biotin deficiency (5,9).

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Effect of Monosodium Glutamate on Blood Ketones in Sheep

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ABSTRACT Experiments were conducted with sheep to evaluate the influence of monosodium glutamate (MSG) on blood ketone levels. The infusion of 0.5 mole of butyric acid per 45.4 kg of body weight into the rumen produced significant increases ($P < 0.05$) in blood ketone levels which were reduced by the administration of MSG. A dose response was obtained as the levels of MSG increased. Significant ($P < 0.05$) reductions in blood ketones occurred at MSG levels starting at 0.6 mole/45.4 kg of body weight. MSG also reduced blood ketones when given as an intravenous injection or when fed in the diet. MSG acted faster than propylene glycol in reducing blood ketones when the comparison was made either on an equal weight or an equal mole basis. The blood glucose response was triphasic when butyric acid was infused into the rumen. This triphasic response was not changed by administering MSG with the butyric acid. MSG, given alone, increased blood glucose indicating that it is glucogenic in the ruminant animal.

The underlying causes of ketosis in ruminants are related to the control and integration of carbohydrate and fat metabolism. Although the knowledge relating to this problem has increased in the past few years (1), there is not complete agreement (2) on an hypothesis which explains the biochemical nature of the disorder. The essential feature in any treatment of ketosis is to supply the animal with energy as glucose or glucose precursors. For example, the intramuscular injection of glucocorticoids or ACTH and the intravenous injection of glucose have been effective treatments (3). Oral administration of sodium propionate or propylene glycol lowers blood ketones and can prevent the ketotic condition (3, 4). It is believed that propylene glycol is converted to lactic acid and subsequently to glucose. Glutamic acid is a potential source of glucose precursors and subsequently glucose. It can be converted readily to α-ketoglutaric acid and then, via the tricarboxylic acid cycle, to oxaloacetate.

The present studies were designed to evaluate the influence of monosodium glutamate (MSG) on the level of blood ketones in the ruminant and to compare the response with that obtained with propylene glycol.

EXPERIMENTAL

Animals. Sheep were used in these studies because of their adaptability to this type of experimentation. The animals, 10 wethers weighing 60 to 70 kg, were used for more than one trial. They were not used for any subsequent treatment for at least 1 week. Prior to each experiment, blood ketones and blood glucose were measured. The diet fed consisted of: (in percent) ground corn cobs, 40; ground corn, 38.65; soybean oil meal (44% protein), 15; dehydrated alfalfa meal (17% protein), 5; iodized salt, 1; calcium phosphate (dibasic), 0.2; vitamin mixture (2250 IU of vitamin A and 400 IU of vitamin D_3 per gram), 0.15.

Procedure. Blood ketone levels were increased by administering a ketogenic fatty acid, butyric acid. It was neutralized to pH 7 with NaOH, diluted with water and administered by stomach tube to supply 0.5 mole/45.4 kg of body weight. MSG or propylene glycol was dissolved in water and administered by the same method. The control animals were dosed with water. MSG was also fed in the diet or given by intravenous injection.

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Blood samples were taken from the jugular vein using 10 ml Vacutainers containing sodium heparin. The samples were obtained before administration of the test materials and at intervals afterward up to 8 hours. Protein-free filtrates of the blood were prepared (5) and blood glucose was determined (6). Blood ketones were distilled (7) and the acetone was determined colorimetrically (8).

The data were analyzed statistically according to the procedures outlined by Steel and Torrie (9). Results from identical trials were combined and summarized for presentation.

RESULTS AND DISCUSSION

Effect of MSG on blood ketone levels. No significant changes occurred in the blood ketone levels of the control sheep throughout the experimental period (table 1). Treating sheep with 0.5 mole of butyric acid per 45.4 kg of body weight caused a significant ($P < 0.05$) and rapid increase in blood ketones. Peak concentrations were reached at 3 hours and then declined gradually toward normal levels. It has been demonstrated by Schultz and Smith (10) that blood ketones can be increased by the administration of butyric acid which is converted by the rumen wall to β-hydroxybutyrate and passed on to the liver in this form. The results obtained in these experiments indicate that this can occur rapidly. The level of blood ketones was three times higher than normal within 15 minutes after placing the butyric acid into the rumen. When MSG was given with butyric acid a rapid and significant ($P < 0.05$) rise in blood ketones occurred; however, 30 minutes after the treatment the blood ketones of the sheep receiving

TABLE 1

Effect of monosodium glutamate (MSG) on blood ketone levels

Time after treatment	Treatment			
	Untreated control [1]	0.5 mole butyric acid [1,2]	0.5 mole butyric acid plus 0.6 mole MSG [1,2]	0.5 mole butyric acid plus dietary MSG [3]
hr	*mg ketones/100 ml blood* [4,5]			
0	2.8 ± 0.2 [a]	3.2 ± 0.2 [a]	3.5 ± 0.3 [a]	2.6 ± 0.1 [a]
0.25	2.9 ± 0.2 [a]	9.1 ± 0.9 [b]	7.9 ± 1.1 [b]	—
0.5	2.6 ± 0.3 [a]	10.5 ± 0.9 [b]	7.9 ± 0.3 [c]	—
1	3.0 ± 0.2 [a]	11.5 ± 0.9 [b]	7.4 ± 0.4 [c]	8.2 ± 0.1 [c]
2	2.7 ± 0.2 [a]	12.7 ± 0.8 [b]	7.0 ± 0.6 [c]	9.8 ± 0.1 [b,c]
3	2.9 ± 0.3 [a]	14.5 ± 0.5 [b]	7.3 ± 0.3 [c]	10.0 ± 0 [d]
4	2.8 ± 0.2 [a]	10.5 ± 0.8 [b]	6.7 ± 0.2 [c]	—
6	2.5 ± 0.2 [a]	6.1 ± 0.5 [b]	5.5 ± 0.3 [b]	4.4 ± 0.1 [b]
8	2.2 ± 0.7 [a]	5.7 ± 1.1 [a]	4.8 ± 0.1 [a]	—

[1] Six to 10 animals included in mean.
[2] Amount administered per 45.4 kg body weight.
[3] Five hundred grams of diet containing 5% MSG; two animals included in mean.
[4] Mean ± SE of mean.
[5] Values followed by the same superscript are not significantly different ($P < 0.05$), based on Duncan's multiple range test.

TABLE 2

Effect of increasing doses of monosodium glutamate (MSG) on blood ketone levels

Time after treatment	Treatment				
	0 mole MSG [1]	0.3 mole MSG [1]	0.6 mole MSG [1]	0.75 mole MSG [1]	0.9 mole MSG [1]
hr	*mg ketones/100 ml blood* [2,3]				
0	3.5 ± 0.4 [a]	4.0 ± 0.4 [a]	4.1 ± 0.2 [a]	3.2 ± 0.1 [a]	3.3 ± 0.7 [a]
1	10.8 ± 1.0 [a]	11.6 ± 0.4 [a]	8.1 ± 0.7 [b]	5.2 ± 0.3 [c]	4.8 ± 0.4 [c]
2	11.6 ± 0.3 [a]	11.9 ± 0.9 [a]	7.8 ± 0.8 [b]	5.1 ± 0.6 [c]	4.9 ± 0.2 [c]
3	12.5 ± 0.8 [a]	11.0 ± 0.3 [a]	7.9 ± 1.1 [b]	5.5 ± 0.8 [c]	4.5 ± 0.4 [c]
6	6.1 ± 0.2 [a]	6.6 ± 0.7 [a]	5.5 ± 0.3 [a,b]	4.5 ± 0.4 [b]	4.5 ± 0.4 [b]

[1] Amount administered per 45.4 kg body weight. All treatments included 0.5 mole of butyric acid per 45.4 kg body weight.
[2] Mean ± SE of mean. Four animals included in each mean.
[3] Values followed by the same superscript are not significantly different ($P < 0.05$), based on Duncan's multiple range test.

0.6 mole of MSG per 45.4 kg body weight were lower than those not receiving MSG. This difference was significant ($P < 0.05$) throughout the first 4 hours after treatment.

Blood ketones were reduced by feeding 500 g of a diet containing 5% MSG. This reduction was not as great as that observed when MSG was infused into the rumen; however, the amount of MSG consumed by the sheep via the diet was one-fifth as much as was given by infusion. No significant reduction in blood ketones occurred by feeding 500 g of a diet containing 1% MSG. Feeding this diet at a rate of 1000 g/day for 6 weeks prior to being challenged with butyric acid (0.5 mole/45.4 kg body weight) did not lower blood ketone levels.

Effect of increasing doses of MSG on blood ketone levels. When sheep were treated with butyric acid or butyric acid plus increasing levels of MSG (0.3, 0.6, 0.75, and 0.9 mole MSG/45.4 kg body weight) a gradient reduction of blood ketones was obtained (table 2). Significant reductions ($P < 0.05$) in blood ketones occurred at MSG levels of 0.6 to 0.9 mole/45.4 kg of body weight.

Effect of intravenous infusion of MSG on blood ketone levels. In a single experiment, MSG was administered by intravenous infusion (fig. 1). One sheep was infused with butyric acid constantly over an 8-hour period through a rumen fistula at a rate of 10 g/hour. The period of time during which the sheep also received MSG intravenously, at a rate of 5.5 g/hour, is indicated on the figure. A continuous drop in blood ketone levels occurred during the MSG infusion even though the animal was also being infused via the rumen with butyric acid. Following the termination of MSG infusion, the blood ketone levels began to rise again. This experiment demonstrated that MSG could lower blood ketones when given intravenously.

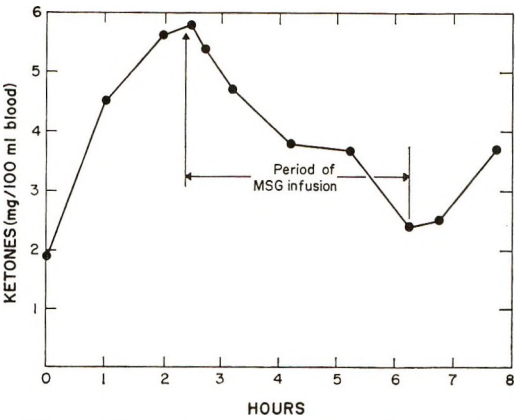

Fig. 1 Effect of intravenous infusion of monosodium glutamate (MSG) on blood ketone levels.

Comparison of MSG and propylene glycol effects on blood ketone levels. Preliminary experiments showed that propylene glycol did not reduce blood ketone levels significantly when given at the same time as butyric acid. Propylene glycol was found to be more effective when given 1 hour before dosing with butyric acid. The rate of propylene glycol absorption through the rumen wall could be one explanation for this effect; however, Emery et al. (4) have reported measurable blood levels within 10

TABLE 3

Comparison of effects of monosodium glutamate (MSG) and propylene glycol on blood ketone levels

Time after treatment	Treatment			
	0.5 mole butyric acid [1]	0.75 mole MSG [1]	57 g MSG [1,2]	0.75 mole propylene glycol [1]
hr	*mg ketones/100 ml blood* [3,4]			
0	2.4 ± 0.2 [a]	2.2 ± 0.2 [a]	1.9 ± 0.2 [a]	2.1 ± 0.2 [a]
1	9.2 ± 0.7 [a]	4.4 ± 0.5 [b]	5.4 ± 0.6 [b]	8.6 ± 0.7 [a]
2	10.9 ± 0.5 [a]	5.1 ± 0.5 [b]	5.8 ± 0.6 [b]	8.5 ± 0.7 [c]
3	11.6 ± 0.5 [a]	6.6 ± 0.4 [b]	6.8 ± 0.8 [b]	7.8 ± 0.6 [b]
6	5.2 ± 0.6 [a]	4.7 ± 0.3 [a]	5.6 ± 0.4 [a]	5.3 ± 0.5 [a]

[1] Amount administered per 45.4 kg body weight. All treatments included 0.5 mole of butyric acid per 45.4 kg body weight.
[2] Weight in grams of 0.75 mole of propylene glycol.
[3] Mean ± SE of mean. Six to 10 animals included in mean.
[4] Values followed by the same superscript are not significantly different ($P < 0.05$), based on Duncan's multiple range test.

TABLE 4

Effect of monosodium glutamate (MSG) and butyric acid on blood glucose levels

Time after treatment	Treatment			
	Untreated control [1]	0.6 mole MSG [2,3]	0.5 mole butyric acid [1,2]	0.5 mole butyric acid plus 0.6 mole MSG [1,2]
hr	*mg glucose/100 ml blood* [4,5]			
0	59.4 ± 1.0 [a]	65.5 ± 0 [a]	62.8 ± 1.4 [a]	60.3 ± 1.2 [a]
0.25	62.3 ± 1.7 [a]	71.5 ± 0.5 [a]	104.3 ± 2.6 [b]	93.3 ± 4.1 [c]
0.5	62.4 ± 1.2 [a]	72.2 ± 1.2 [b]	87.0 ± 3.5 [c]	66.9 ± 1.6 [a,b]
1	61.8 ± 1.1 [a,b]	75.5 ± 0.5 [a]	55.1 ± 3.4 [b]	45.5 ± 2.3 [c]
2	62.4 ± 1.3 [a]	69.0 ± 1.0 [a]	50.1 ± 3.2 [b]	51.4 ± 1.8 [b]
3	63.0 ± 1.6 [a]	71.5 ± 0.5 [a]	55.9 ± 2.4 [b]	55.7 ± 1.6 [b]
4	60.6 ± 0.7 [a]	69.0 ± 1.0 [a]	61.7 ± 2.3 [a]	58.1 ± 2.3 [a]
6	60.1 ± 1.7 [a]	70.0 ± 0 [b]	68.1 ± 2.4 [b]	63.7 ± 1.4 [a,b]
8	58.4 ± 1.6 [a]	—	71.3 ± 1.2 [b]	63.9 ± 2.0 [c]

[1] Sixteen animals included in mean.
[2] Amount administered per 45.4 kg body weight.
[3] Two animals included in mean.
[4] Mean ± SE of mean.
[5] Values followed by the same superscript are not significantly different ($P < 0.05$), based on Duncan's multiple range test.

minutes after its administration into the rumen. A more plausible explanation is that the maximum conversion of propylene glycol to blood glucose does not occur until 3 hours after a single intraruminal dose (11).

Monosodium glutamate was more effective than propylene glycol in reducing blood ketones during the first 2 hours after butyric acid treatment (table 3). These differences were significant ($P < 0.05$) when based on an equal molar as well as an equal weight comparison of MSG to propylene glycol.

Effect of MSG and butyric acid on blood glucose levels. Changes in the concentration of blood glucose occurred following treatment with butyric acid, MSG, or butyric acid plus MSG (table 4). No significant changes in blood glucose levels occurred in the control animals. A rise in blood glucose was noted after treating with MSG and the elevated level was maintained for the duration of the experiment. The blood glucose response was triphasic when sheep were dosed with butyric acid. This was characterized by an initial increase, then a decrease, and finally an increase to higher than normal levels. This has been observed by others (10) and has been explained as a combination of insulin release (12) [2] glycogenolysis (13) and glucose sparing (14).

Under the experimental conditions of these studies, MSG was effective in reducing blood ketone levels when administered via the diet, placed directly into the rumen, or given intravenously. MSG also produced a faster response than propylene glycol in lowering induced blood ketone levels.

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