Sodium Metabolism and Requirements in Lactating Rats [1,2]

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ABSTRACT Sodium metabolism was studied in 4 groups of 11 lactating rats fed a diet containing either 0.03, 0.05, 0.07 or 0.09% sodium. Less feed intake, a loss in body weight and smaller pups at weaning were found with the group fed the 0.03% sodium compared with the 3 higher sodium groups. Plasma and bone sodium were lowest in the 0.03% sodium group following lactation, whereas no differences were found among the 3 higher sodium groups. There were no differences in blood cell volume or bone moisture among the groups. Milk samples taken on day 22 of lactation showed a higher fat content for the 0.03% sodium group, whereas the sodium concentration was significantly lower for the 0.03% than for the 0.09% group. Adrenal zona glomerulosa areas were largest for the 0.03% group, next largest for the 0.05% group and smallest for the 0.07 and 0.09% groups. Kidney juxtaglomerular granulation was greatest in the rats fed 0.03% sodium. With this nonpurified diet, 0.05% sodium was adequate for rats nursing 9 pups.

The sodium requirements of rats during gestation and lactation have been estimated to be 0.5% and 0.3% of the diet, respectively (1). These estimates were derived from data obtained in the 1920's and appear to be unreasonably large, especially in relation to the estimated sodium requirement for growth (0.05%). A reinvestigation of the sodium requirement for gestation showed that the older estimate was indeed excessive (2). Thus it seemed worthwhile to continue the study to confirm or refute the estimate of sodium required for lactation and to obtain more information about sodium metabolism in the lactating rat.

PROCEDURE

The present study is a continuation of the previous work (2) on sodium metabolism during reproduction. Eleven rats were continued through lactation at each of the following dietary sodium levels: 0.03, 0.05, 0.07 and 0.09%. None of the pups born to the females fed 0.01% sodium survived more than 24 hours; consequently, this diet was not included in the lactation study. The diets were identical with those of the previous study (2) and the rats were kept in the same wire mesh cages in which they had littered with sawdust maintained as bedding. The body weights of the females as well as the litters were recorded every third day during the 21-day lactation period. Litter sizes were adjusted to 9 pups on the third day after birth, whenever necessary. The females were milked by hand on day 22 of lactation. The rat bellies were shaved prior to milking to prevent contamination of the milk. Pentobarbital sodium (6.5 mg) as well as oxytocin (3 USP units) was administered to facilitate milking. Milk fat was determined by a microcentrifugation method (3). Milk sodium was determined spectrophotometrically on duplicate 0.5 ml samples of milk which were dry ashed, dissolved in dilute hydrochloric acid and made to volume with water.

Plasma sodium concentrations and hematocrit readings were determined with duplicate blood samples of about 0.1 ml taken from the cavernous sinus (4) on day 22 of lactation.

Following collection of blood samples, the rats were killed and the femurs, kidneys and adrenals were removed immediately. The procedures employed for analy-

Received for publication July 22, 1969.
[1] Paper No. 6981, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minnesota.
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ses of the bones and histological preparation of the kidneys and adrenals were those used in the previous study (2). Differences among the treatment means were assessed in accordance with Duncan's new multiple range test (5).

RESULTS AND DISCUSSION

The maternal body weight changes and feed intakes as well as the body weights and numbers of pups at weaning are given in table 1. Normal changes in body weight during lactation are reported to be ± 10 g (1). The rats fed the 0.03% sodium diet lost body weight in excess of this amount, ate less feed than the rats fed more sodium and weaned pups which were distinctly lighter than pups in the other groups. If the weaning weights of the pups were influenced primarily by the milk production of the dams, then the 0.03% sodium diet presumably did not allow as much milk production as the higher sodium diets. Animals producing less milk would be expected to require less nutrients and therefore eat less.

The excessive body weight loss of the rats fed 0.03% sodium may indicate that body sodium was depleted for milk production. Mature rats have been shown to exhibit a distinct loss of body weight in response to acute sodium depletion (6). The mobilization of nutrients when body tissues are catabolized would be another factor for negatively influencing feed intake. Lactating dairy cows fed diets without supplemental salt also show symptoms which include loss of body weight, reduction of appetite and decreased milk production (7, 8).

The maternal weight changes, feed intakes and pup weaning weights for the rats fed the diet containing 0.05% sodium were within the range of values obtained in this colony with stock diets. Groups fed the diets containing more sodium did not show significant improvement.

The progression of feed intake and maternal body weight changes during lactation is illustrated by figure 1.

Table 2 shows the mean hematocrit values and plasma sodium concentrations at the termination of lactation. The plasma sodium concentration was reduced in rats fed 0.03% sodium but the hematocrits showed no differences among dietary groups. Plasma sodium concentrations did not increase above the values found with 0.05% sodium in those rats fed more sodium.

Comparison of the plasma sodium and hematocrit values with the corresponding values determined immediately prior to parturition (2) indicated that the blood cell volume increased but plasma sodium decreased in all groups during lactation. To ascertain the effects of the milking procedures on plasma sodium and hematocrit values, a separate small trial was conducted with 5 lactating rats fed a stock diet. The milking procedures of the main experiment were followed exactly but blood samples were taken both before and after milking. The mean hematocrit values were 54.0 and 60.0% while the corresponding plasma sodium concentrations were 142.8 and 134.7 mEq/liter before and after milking, respectively. The differences in pre- and post-lactation values were therefore largely or entirely due to the milking procedures.

The bone sodium and moisture data are presented in table 3. Bone sodium was less in the femurs from rats fed the 0.03%

TABLE 1

Maternal body weight change and feed intake during lactation, number of pups weaned and body weight of pups at weaning

Dietary sodium	Mean body weight after parturition	Change in body weight [1,2]	Total feed intake [1,2]	No. of live pups at weaning [1,2]	Weaning weights of pups [1,2]
%	g	g	g		g
0.03	250.3	−20.7 ± 4.8 [a]	666.0 ± 18.3 [a]	8.3 ± 0.29	26.0 ± 1.1 [a]
0.05	272.0	3.8 ± 5.3 [b]	784.1 ± 23.6 [b]	8.4 ± 0.33	35.3 ± 1.3 [b]
0.07	273.8	8.3 ± 4.1 [b]	830.9 ± 26.2 [b]	8.3 ± 0.39	38.7 ± 1.4 [b]
0.09	278.1	3.8 ± 4.3 [b]	829.0 ± 28.6 [b]	8.7 ± 0.14	37.1 ± 1.7 [b]

[1] Mean ± SEM, 11 rats per group.
[2] Values with unlike superscripts are significantly different, $P < 0.01$.

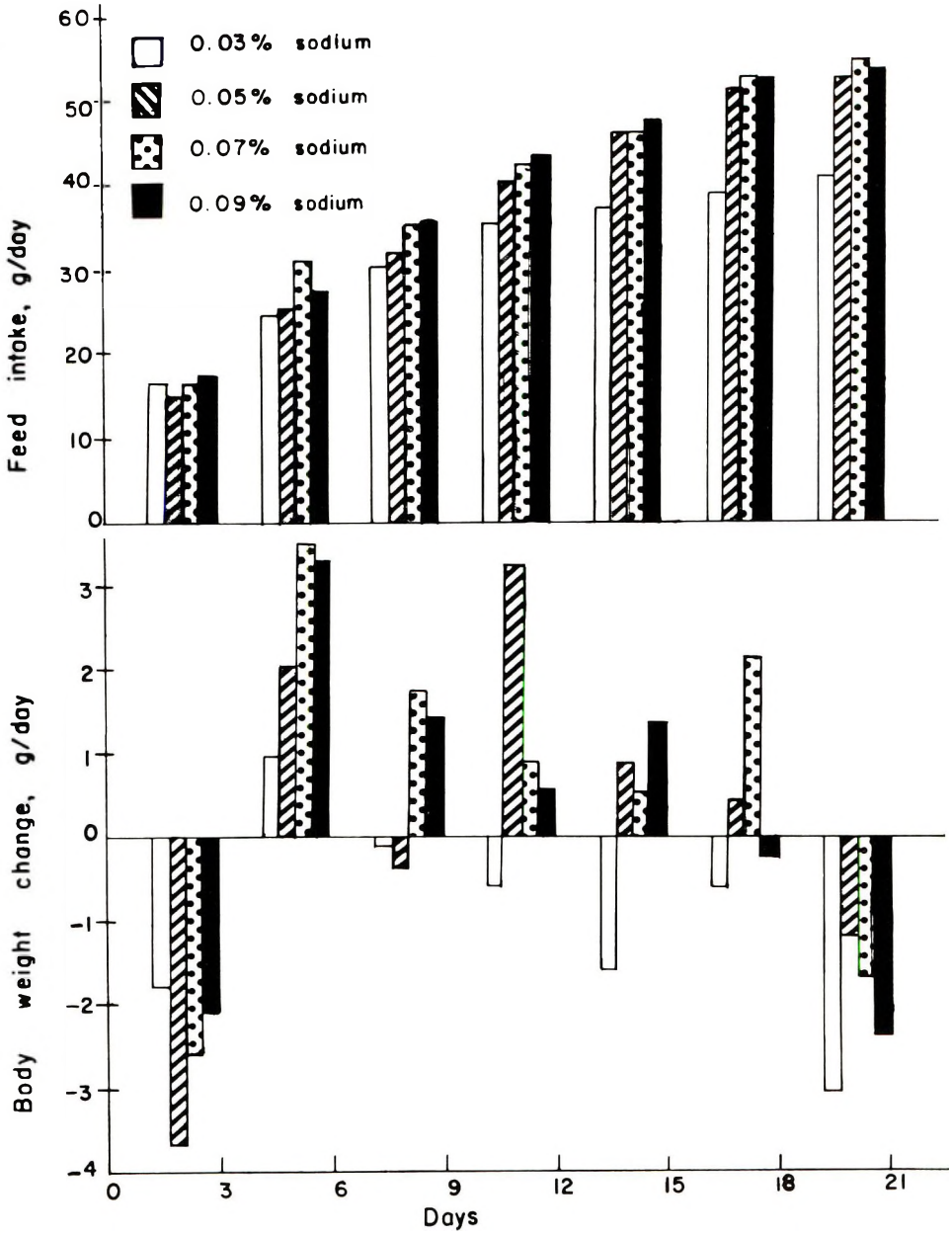

Fig. 1 Maternal body weight changes and food intakes measured at 3-day intervals during lactation.

sodium diet but no other differences were found. There was no detectable influence of dietary sodium on bone moisture. In rats and in man, the skeleton contains about 40% of the total body sodium (9, 10). This bone sodium consists of a nonexchangeable as well as an exchangeable fraction (11, 12) and in the rat the proportion of the latter decreases with age (13). The bone sodium concentrations, shown in table 3, follow a pattern very similar to the plasma sodium concentrations given in ta-

TABLE 2

Hematocrit and plasma sodium concentrations of maternal rats on day 22 of lactation

Dietary sodium	Hematocrit [1,2]	Plasma sodium [1,2]
%	%	*mEq/liter*
0.03	63.9 ± 0.91	121.0 ± 1.19 [a]
0.05	63.3 ± 0.68	130.6 ± 0.97 [b]
0.07	63.8 ± 0.94	129.2 ± 1.00 [b]
0.09	63.6 ± 1.00	131.4 ± 0.76 [b]

[1] Mean ± SEM, 11 rats per treatment.
[2] Values with unlike superscripts are significantly different, $P < 0.01$.

TABLE 3

Bone moisture and sodium concentrations of maternal rats on day 22 of lactation

Dietary sodium	Moisture [1,2]	Sodium [1,2]
%	%	*mEq/kg dry bone*
0.03	13.0 ± 0.22	256.5 ± 2.2 [a]
0.05	13.2 ± 0.12	265.6 ± 1.8 [b]
0.07	13.1 ± 0.18	264.1 ± 1.4 [b]
0.09	13.2 ± 0.17	265.0 ± 1.2 [b]

[1] Mean ± SEM, 11 rats per treatment.
[2] Values with unlike superscripts are significantly different, $P < 0.01$.

ble 2. Extrapolation of the reduction in bone sodium per reduction in plasma sodium leads to an estimate that roughly one-half of the bone sodium is in equilibrium with extracellular-fluid sodium.

The rats fed the least sodium produced milk with nearly a third more fat than was found in the milk of other groups. This was the only difference found in milk fat among the groups. The group means for fat and sodium in the milk are given in table 4. A mechanism whereby low dietary sodium leads to an increased proportion of fat in the milk is not known. It is possible, although not probable, that the higher milk fat was associated with more complete milking in this group. Some difficulty was experienced in obtaining milk samples of adequate amount from this group but all rats were milked out as far as possible. Rat milk obtained in the latter stages of lactation has been reported to contain an average of 10.5% fat and 0.14% (61 mEq/liter) sodium (14).

The sodium content of milk is expressed on the basis of fat-free as well as whole milk in table 4 because the fat-free basis

TABLE 4

Fat and sodium content of rat milk on day 22 of lactation

Dietary sodium	Fat [1]	Sodium [1]	
		Whole milk	Fat-free milk
%	%	*mEq/liter*	*mEq/liter*
0.03	13.3 ± 0.88 [a]	57.6 ± 2.0 [e]	66.4 ± 2.2 [e]
0.05	10.0 ± 0.35 [b]	61.7 ± 2.3 [e,f]	68.6 ± 2.6 [e,f]
0.07	10.4 ± 0.38 [b]	62.7 ± 2.5 [e,f]	69.9 ± 2.6 [e,f]
0.09	10.5 ± 0.68 [b]	65.8 ± 2.8 [f]	73.6 ± 3.0 [f]

[1] Mean ± SEM, 11 rats per treatment. Values with superscripts are significantly different, $P < 0.01$, and values with subscripts are significantly different, $P < 0.05$, only when the same letter does not appear in the superscript or subscript.

TABLE 5

Measures of adrenal zona glomerulosa development and kidney juxtaglomerular cell granulation from maternal rats on day 22 of lactation

Dietary sodium	No. of rats	Total area [1]	Cells in area [1]	Area per cell [1]	Juxtaglomerular index [1]
%		mm^2		μ^2	
0.03	5	1.90 ± 0.09 [a]	11515 ± 497 [e,a]	165.2 ± 4.3 [a]	33.5 ± 3.3 [e]
0.05	5	1.30 ± 0.07 [b]	9590 ± 397 [e,a,b]	136.0 ± 3.1 [b]	27.8 ± 2.7 [e,f]
0.07	6	1.01 ± 0.05 [c]	8475 ± 455 [f,b]	119.5 ± 3.4 [c]	24.2 ± 1.3 [f]
0.09	7	0.85 ± 0.06 [c]	7501 ± 486 [f,b]	113.8 ± 2.8 [c]	22.4 ± 2.0 [f]

[1] Mean ± SEM. Values with superscripts differ significantly, $P < 0.01$, and values with subscripts differ significantly, $P < 0.05$, only when the same letter does not appear in the superscript or subscript.

seems more reasonable for the estimation of treatment effects. Statistical analysis showed that the sodium concentration was less in milk from the 0.03% sodium group than from the 0.09% sodium group when calculated on either the whole or fat-free basis. No other differences were significant although there appears to be a trend for milk sodium to increase with dietary sodium.

The results of the histological studies of the kidneys and adrenals are shown in table 5. The area of the zona glomerulosa decreased progressively with increasing dietary sodium to 0.07% but no further significant decrease in area was observed at 0.09% sodium. The number of cells comprising the glomerulosa area also decreased with increasing dietary sodium, similar to the area itself but significant differences were not demonstrated between the 0.03 and 0.05% groups. In addition, the average individual cell area decreased with increasing dietary sodium in the same pattern as the glomerulosa area. The increase in size of the zona glomerulosa which occurred with decreased sodium in the diet was, therefore, accomplished through both hyperplasia and hypertrophy.

Comparison of glomerulosa areas measured after lactation with the similar values obtained immediately following parturition (2) shows that the area increased during lactation in the rats fed 0.03 and 0.05% sodium. No significant changes were seen to result in cell number or individual cell areas, although the tendency appears to have been for both to increase.

The granulation of juxtaglomerular cells in the kidneys as assessed by the juxtaglomerular indices was greatest in the rats fed 0.03% sodium, and no significant differences were detected among the other groups. A comparison of the indices determined at the end of the lactation with those obtained just prior to lactation (2) shows that an increase in granulation occurred during lactation in rats from the 0.05% groups only. This result is attributed primarily to the deviant granulation value found in this group after parturition. The mean indices were very similar before and after lactation for the 0.07 and 0.09% sodium groups.

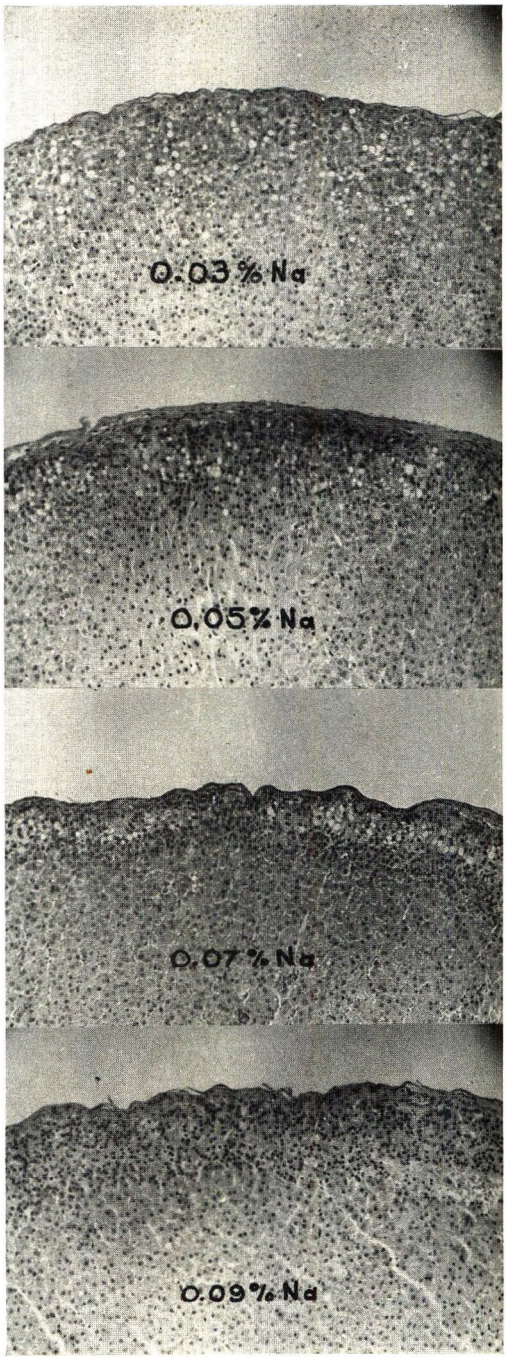

Fig. 2 Representative sections of the adrenal zona glomerulosa at the end of lactation. Magnification 425 ×, stained with hematoxylin and eosin.

When the relationship between the juxtaglomerular indices and the zona glomerulosa areas was examined, a significant linear correlation ($r = 0.68$) was found. This was similar to results which had been obtained previously (2, 15).

The histological data show a trend toward relaxation of the sodium conservation mechanism with each increase in dietary sodium, although significant differences between the two highest sodium diets were not demonstrated. Comparison of these measures with similar data obtained just prior to lactation indicates that lactation sodium requirements are greater than gestational requirements.

The greater area of zona glomerulosa in the 0.05% group was accompanied by a small amount of cytoplasmic vacuolation in the area and a slight amount of this vacuolation was also noted in the adrenal of the 0.07% group. Representative adrenal sections for the dietary groups are shown in figure 2. There was no evidence in this study to indicate that this vacuolation was detrimental and it had been previously shown by others to be reversed within 4 days of feeding a sodium load (16).

The sodium status of the 0.03% sodium group was slightly suboptimal at the start of lactation. This level of sodium, however, did not meet the requirement for lactation as shown by excessive maternal weight losses, subnormal growth of pups, reduced plasma and bone sodium and low sodium content of milk. These measures were all satisfactory when the diet contained 0.05% sodium, and this level was considered adequate; however, to avoid adrenal and kidney changes the 0.09% sodium diet would be necessary. Slightly greater levels would be required with purified diets.

LITERATURE CITED

1. National Research Council 1962 Nutrient requirements of domestic animals: Nutrient requirements of laboratory animals. National Academy of Sciences–National Research Council Publ. 990.
2. Ganguli, M. C., J. D. Smith and L. E. Hanson 1969 Sodium metabolism and its requirement during reproduction in female rats. J. Nutr., *99:* 225.
3. Ganguli, M. C., J. D. Smith and L. E. Hanson 1969 Indirect micro-method for milk fat determination. J. Dairy Sci., *52:* 126.
4. Lapeyrac, F. 1963 Blood sampling from the cavernous sinus. Rev. Fr. Etud. Clin. Biol., *8:* 195.
5. Duncan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics, *11:* 1.
6. Baker, G. P., H. Levitin and F. H. Epstein 1961 Sodium depletion and renal conservation of water. J. Clin. Invest., *40:* 867.
7. Babcock, S. M. 1905 The addition of salt to the ration of dairy cows. Wisc. Agr. Exp. Sta. Rep. no. 22: 129.
8. Smith, S. E., and P. D. Aines 1959 Salt requirements of dairy cows. Cornell Univ. Agr. Sta., New York, Bull. no. 938.
9. Nichols, G., Jr., and N. Nichols 1956 The role of bone in sodium metabolism. Metabolism, *5:* 438.
10. Norman, N. 1963 The participation of bone in the sodium and potassium metabolism of the rat. I. Simultaneous determination of the exchangeable body sodium and potassium, and the exchangeable and inexchangeable fractions of these ions in bone and in the normal rat. Acta Physiol. Scand., *57:* 363.
11. Bauer, G. C. H. 1954 Metabolism of bone sodium in rats investigated with $sodium^{22}$. Acta Physiol. Scand., *31:* 334.
12. Bergstrom, W. H. 1956 The skeleton as an electrolyte reservoir. Metabolism, *5:* 433.
13. Forbes, G. B., G. Mizner and A. Lewis 1957 Effect of age on radiosodium exchange in bone (rat). Amer. J. Physiol., *190:* 152.
14. Lukey, T. D., T. J. Mande and J. Pleasants 1954 The physical and chemical characterization of rat's milk. J. Nutr., *54:* 345.
15. Wardlaw, J. M., and R. L. Pike 1963 Some effects of high and low sodium intakes during pregnancy in the rat. IV. Granulation of renal juxtaglomerular cells and zona glomerulosa width. J. Nutr., *80:* 355.
16. Pike, R. L., J. E. Miles and J. M. Wardlaw 1966 Juxtaglomerular degranulation and zona glomerulosa exhaustion in pregnant rats induced by low sodium intakes and reversed by sodium load. Amer. J. Obstet. Gynecol., *95:* 604.

Effect of Maternal Protein Deprivation on Morphological and Enzymatic Development of Neonatal Rat Tissue [1,2]

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ABSTRACT Tissues of neonatal young of female rats maintained throughout pregnancy on diets containing either 6% or 30% casein as the sole source of protein were studied to determine the effect of maternal protein deprivation on prenatal development. Routine H and E-stained sections were studied for evaluation of morphology, and recognized histochemical procedures were used for the detection of SDH, MDH, LDH, G-6-PDH, DPN and TPN diaphorases, acid and alkaline phosphatases, ATPase, cytochrome and monoamine oxidases, nonspecific esterase, leucine aminopeptidase and γ-glutamyltranspeptidase. Although all young of females fed the low protein ration were reduced in size, only those whose birth weights were less than 4.5 g showed morphological or enzymatic abnormalities. In brain, spinal cord, pancreas, skin and intestine, cellular and enzyme changes were observed. Abnormal enzyme patterns were observed in thyroid, paravertebral ganglia and choroid plexus of low protein young. Liver and brown fat cells of these animals contained reduced enzymatic activity and increased amounts of intracellular fat. Alkaline phosphatase activity appeared to be related to altered cellular maturation in several tissues of low protein neonatal rats.

It has previously been demonstrated that maternal protein deficiency in the rat results in the production of young that are smaller than normal at birth (1). The decrease in size of the liver, kidney, heart, thymus, brain and carcass which occurs in these animals is the result of a decrease in the total numbers of cells in these organs and tissues (1).

Organ size in developing animals has been shown to be related to the degree of maturation (2, 3), suggesting that various tissues and organs in the young of animals fed a protein-deficient diet during pregnancy may be immature. Studies have revealed that morphological retardation (4) and altered functional capacity (5) can be demonstrated in the kidneys of neonatal and early postnatal rat pups. The work reported here was undertaken to investigate the effect of maternal protein intake on the morphological development of the remaining organs and tissues of neonatal animals. Evidence of the comparative biochemical maturity (6) of neonatal control and low protein rats was obtained from histochemical studies of their enzyme patterns.

MATERIALS AND METHODS

Pregnant female rats of the Sprague-Dawley strain were maintained throughout pregnancy on diets containing either 30% or 6% casein as the sole source of protein as previously described (1). Fifty-seven full-term young, obtained from 22 litters of low protein-fed females, and 86 young from 31 control litters constituted the experimental material for the present study. At the commencement of parturition, the maternal animals were killed by decapitation and the young were removed from the uterus. Tissues to be processed for enzyme studies were rapidly dissected and frozen in isopentane which had been chilled in liquid nitrogen. Tissues obtained from littermates were fixed in Bouin's solution and were subsequently embedded in paraffin, sectioned at 6 μ, stained with hematoxylin and eosin, and studied microscopically for details of cellular morphology.

Received for publication June 9, 1969.
[1] Supported in part by Grant no. 375 from The Nutrition Foundation, Inc.
[2] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1969.

The tissues examined included adrenal, bone, brain, brown fat, cartilage, esophagus, heart, intestine, kidney, liver, lung, pancreas, parathyroid, paravertebral ganglia, salivary glands, skeletal muscle, skin, spinal cord, spleen, stomach, thymus, thyroid and trachea. All histochemical enzyme procedures were done on unfixed, frozen cryostat sections, mounted on clean coverslips and air dried.

The enzymes demonstrated histochemically were: acid phosphatase (7),[3] alkaline phosphatase (7), ATPase (8), succinic dehydrogenase, malic dehydrogenase, lactic dehydrogenase, and glucose-6-phosphate dehydrogenase (9), DPN and TPN diaphorases, and cytochrome oxidase (10), monoamine oxidase (11), nonspecific esterase (9), leucine aminopeptidase (12), and γ-glutamyltranspeptidase (13). Positive control tissues and negative control procedures were included in every run. Methyl green was used as a nuclear counterstain for all procedures with the exception of those for the demonstration of nonspecific esterase where its presence was found to mask the reaction product. Polyvinylpyrrolidone or glycerin was employed as a coverslipping medium. All tissues were processed, mounted, examined and coded on the day they were obtained.

Histochemical enzyme procedures were also done on selected tissues of normal 18- to 21-day fetal rats to provide information on enzyme occurrence and distribution during prenatal development.

RESULTS

Early in the course of this investigation it was noted that there was a marked variation in the response of female rats to protein deprivation during gestation. Some females showed a marked weight loss during the last few days of pregnancy; their coats became rough and matted, their eyes and ears pale in color, and their activity noticeably decreased. Young produced by these females were smaller than control young. Birth weights averaged 4.5 g as compared with an average neonatal weight of 5.9 g for control rats. Microscopic examination of the tissues of these young revealed no morphological or enzymatic abnormalities. Other female rats showed only slight to moderate weight loss and a

[3] Abbreviations used in this paper to indicate enzymes are as follows: acid phosphatase, Ac. P'ase; alkaline phosphatase, Alk. P'ase; succinic dehydrogenase, SDH; malic dehydrogenase, MDH; lactic dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G-6-PDH; DPN diaphorase, DPN diaph.; TPN diaphorase, TPN diaph.; cytochrome oxidase, Cytox; monoamine oxidase, MAO; nonspecific esterase, NS Est.; leucine aminopeptidase, LAP; γ-glutamyltranspeptidase, γ-GTP.

All black-and-white illustrations were made of paraffin-embedded tissues sectioned at 7 μ and stained with hematoxylin and eosin.

Fig. 1 The cells of the cerebral cortex of the normal neonatal rat. × 375.

Fig. 2 A comparable section through the cerebral cortex of the newborn young of a protein-deficient rat. × 375.

Fig. 3 A villus with its crypt (arrow) in the duodenal mucosa of a normal newborn rat showing the characteristic granular cytoplasm of the epithelial cells, the brush border present on the luminal surface of the enterocytes, and the loosely arranged fibers and vascular structures of the lamina propria. × 375.

Fig. 4 Villi in the duodenum of newborn young of a protein-deficient rat showing the reduced size of the villi and the compact character of the core of lamina propria. The enterocytes lack both cytoplasmic granules and brush borders and show a perinuclear clear area. × 375.

Fig. 5 Jejunal villi of the normal newborn rat. × 375.

Fig. 6 Jejunal villi of the intestine of the neonatal young of a protein-deficient rat. These structures are smaller, shorter, and fewer in number than in control tissues, and the enterocytes show changes comparable to those observed in the duodenal epithelium. × 375.

Fig. 7 Villi from the ileum of the normal newborn rat. × 375.

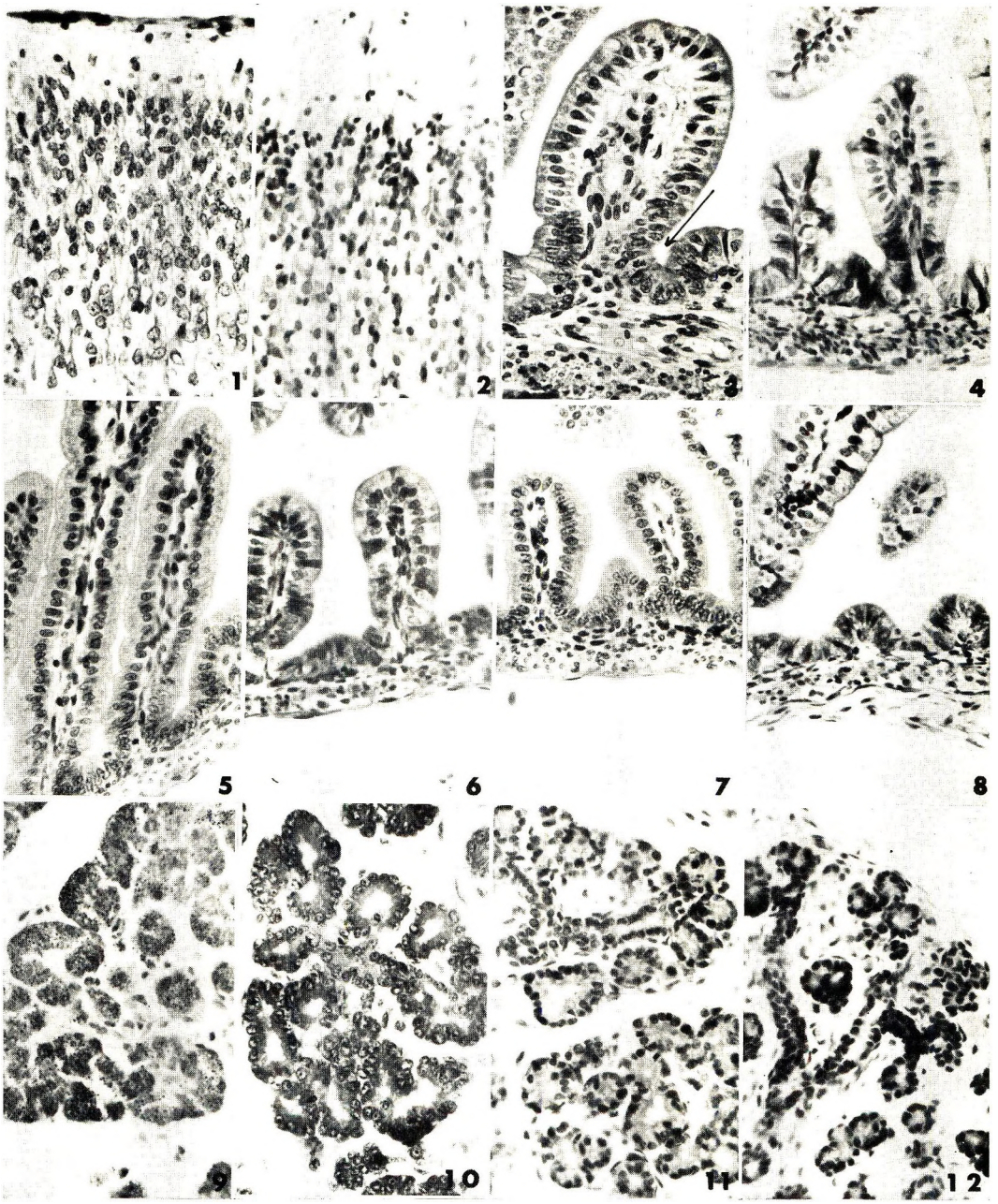
Fig. 8 Primitive villi of the ileum in the intestine of offspring of a protein-deficient rat. × 375.

Fig. 9 Acinar tissue in the pancreas of the normal newborn rat. × 375.

Fig. 10 Pancreatic tissue of the newborn offspring of a protein-deficient rat. The branching intralobular ducts are readily seen in this tissue. The small intensely acidophilic acinar cells are cuboidal rather than pyramidal in shape and are compactly arranged around distended lumina. × 375.

Fig. 11 Submaxillary gland tissue of normal newborn rat. × 375.

Fig. 12 Submaxillary gland tissue of newborn young of a protein-deficient rat. The ductile elements are less numerous, more intensely stained and many of the terminal tubules do not contain distinguishable lumina. × 375.



Figures 1 to 12

minimal dulling and roughness of the coat. Their young were much smaller at term (average weight 3.2 g), and histological and histochemical studies of these organs and tissues revealed the alterations to be described in this paper.

Morphological observations. All organs and tissues of the newborn low protein rat were decreased in size. Where size reduction was present without concomitant alterations in cellular organization or morphology, tissues were considered to be histologically normal. Tissues in which histological changes were found will be discussed in detail.

Brain and Spinal Cord: In both brain and spinal cord of newborn low protein rats, there appeared to be a reduction in the number of large multipolar neurons. The neurons which had differentiated in these tissues did not differ in location, size, or appearance from those in normal full-term young. In the brain there also appeared to be a reduction in the number of cells in the subsurface layers of the cerebral cortex (figs. 1 and 2) and in the granular and Purkinje cell layers of the cerebellar cortex. Under identical conditions of processing, the cell structure in the brains of low protein young was much less well preserved. The special techniques necessary for a more precise study of neurological histology were not applied in the present investigation and further evaluations of these tissues are being made. No gross deformities of brain or spinal cord were observed, and all portions of the choroid, ventricular system, and ependyma appeared normal.

Intestine: The small intestine of the low protein rat pups appeared to be severely affected by maternal protein deprivation. All segments of the gut in these newborn animals contained fewer and less well differentiated villi than were present in comparable segments of the normal newborn intestine. In the duodenum, the changes were not as marked as in succeeding portions of intestine, but even at this level, villi were more widely spaced and shorter, and their enterocytes lacked brush borders in all except a few apically located cells. No crypts of Lieberkühn or Brunner's gland anlagen were present in the duodenum of low protein rat pups, whereas these structures are identifiable in newborn control tissue (figs. 3 and 4).

In the succeeding segments of the intestine, villi were found to be progressively more slender, shorter, and less numerous. The surface epithelium of the jejunal villi was a simple columnar layer which contained far less than the normal number of goblet cells. The enterocytes did not have brush borders on their luminal surfaces, and crypt cells could not be distinguished from cells of the lateral wall of the villus (figs. 5 and 6). Many of the villiform projections were misshapen, paddle-like structures with slender stalk-like bases. In the ileum, villi were very rudimentary in structure and much reduced in number. In many instances, potential villi were present only as epithelial buds without a connective tissue core (figs. 7 and 8).

The colonic mucosa of low protein rat pups showed the invaginated folds characteristic of the normal newborn, but the epithelium covering these folds consisted of low stratified columnar cells and showed a reduction in the customary number of goblet cells. The muscle and connective tissue layers of the intestinal wall were much reduced in thickness in the low protein young but showed no other obvious abnormalities.

Liver: No abnormalities of structure were observed in the livers of low protein rat pups. However, hepatocytes in these tissues were found to contain excessive amounts of fat when compared with those of normal neonatal animals. Decreased numbers of hematopoietic cells were present in the livers of low protein young, but the cells which were present appeared to be normal in type and location. No abnormalities of Kupffer's cells, mitotic activity of hepatocytes, or of vascularity were observed in low protein liver tissue.

Pancreatic Acinar Tissue: The acinar tissue of the pancreas in newborn low protein rats appeared to be reduced in amount as compared with that present in control animals. Acinar units were less densely packed within the lobules, and more intralobular ductile elements were visible than in normal neonatal pancreas (figs. 9 and 10). The small nongranular, intensely acidophilic acinar cells of the low protein tissue were oriented around lumina which

could be seen to be in direct continuity with the branched ducts. Islet tissue appeared not to have been as severely affected by the protein deficit since large and small islets of seemingly normal structure were found throughout the pancreatic tissue.

Submaxillary Gland: Limitation of development was observed in the submaxillary gland tissue of low protein young. The primary and secondary branching ducts and terminal tubules characteristic of this tissue in normal newborn rats were present in low protein young, but these various glandular elements were reduced in both number and size. The terminal tubules, in particular, seemed to be affected, being shorter and less branched than those of control gland tissue. The solid apical cell clusters of intensely stained cells which represented the terminal ends of these structures frequently lacked a central lumen. The cytoplasm of the cells lining the various segments of the ducts was more intensely acidophilic and nuclei were more compactly arranged than in the tissues of control animals (figs. 11 and 12).

Skin: The major change observed in the skin of the neonatal low protein rats was the retardation of development of hair. In the skin of control newborn, hair primordia were densely arrayed in the skin of the head and neck, and both pellage hairs and vibrissae were well differentiated. Many of the larger pellage hairs, and all of the vibrissae had erupted and structurally resembled adult hairs. Sebaceous gland primordia were present at the dermal-epidermal margin of these hairs. In low protein young, vibrissae were smaller than normal, but structurally similar. Pellage hairs, however, were reduced in number and much delayed in development. The epidermis of low protein young was occasionally seen to lack a granulosum and corneum. Although frequent, this alteration was not a consistent finding. No other abnormalities were noted in the cells or tissues of the skin of low protein rat pups.

Histochemical enzyme reactivity. The majority of the morphologically normal cells and tissues of newborn low protein rat pups showed normal histochemical enzyme activity with all of the reactions examined. Brown fat, paravertebral ganglia and thyroid, however, in which no morphological abnormalities had been observed, did display altered enzyme reactivity. Brain, spinal cord and submaxillary gland tissues in which histological evidence of developmental retardation was seen, showed no detectable change in enzyme distribution or reactivity. Other organs, such as intestine, liver, acinar pancreas, and skin, were both morphologically and enzymatically abnormal.

A comparison of enzyme activity in the cells of low protein and control rat pups is presented in table 1. To simplify this chart, only those organs in which morphological or enzyme abnormalities were found have been listed. The alteration of activity observed has been characterized as an increase, a decrease or an absence of demonstrable enzyme in the tissues of low protein pups as compared with controls. The altered enzyme activity of each tissue will be discussed in detail.

Brain and Spinal Cord: The neurons of the macrocellular nuclei of the midbrain, cerebellar Purkinje cells, and the ventral horn cells of the spinal cord, although reduced in number, appeared to be normal in enzymatic activity. They exhibited normally intense reactions for Ac. P'ase, ATPase, SDH, MDH, LDH, G-6-PDH, DPN and TPN diaphorases and NS Est., all of which are normally present in these cells (figs. 13 and 14). The cells of the paravertebral ganglia, which did not appear to be morphologically abnormal or to be reduced in number, were nevertheless enzymatically less reactive in low protein tissues than in their normal counterparts (table 1). In ganglion cells of low protein young, Cytox, MAO and NS Est. which are present in normal ganglion cells were not detectable, whereas Ac. P'ase, ATPase, SDH, MDH, LDH, and DPN and TPN diaphorases were present in reduced amounts (figs. 15 and 16). G-6-PDH appeared to be virtually absent from some of the ganglion cells and present in nearly normal concentration in others. The normal-appearing tissues of the choroid plexus of low protein animals were found to have more intense reactivity of Alk. P'ase and ATPase, reduced activity of Ac. P'ase, SDH, MDH, LDH, and DPN and TPN diaphorases, and to contain no G-6-PDH.

Brown Fat: The brown fat tissue of newborn low protein rat pups did not appear to be diminished in amount, nor to have any cellular abnormalities; however, in enzyme preparations the cells were found to contain demonstrably decreased amounts of all of the oxidative enzymes normally present in control brown fat tissue (figs. 17 and 18). MAO and indoxyl esterase were not demonstrable in brown fat cells from low protein pups, whereas cells containing Alk. P'ase, normally not present in brown fat cells after day 20 of gestation, were numerous in brown fat tissue from low protein young.

Intestine: Intestinal epithelial enzyme activity of protein-deficient rats at term, as revealed by histochemical techniques, correlated well with the severity of the morphological alterations present in the various portions of the gut. In the duodenum, where the more differentiated epithelia were found, enzyme activity most closely resembled the normal pattern; however, both intensity of activity and cellular localization were altered even in this portion of the intestine. Alk. P'ase, for example, was present only in the cells on the apices of the villi (figs. 19 and 20), whereas Ac. P'ase was virtually absent from all duo-

Fig. 13 DPN diaphorase reactivity of the neurons of a macronucleus in the brain of the normal newborn rat. The localization of the enzyme in coarse granules within the cytoplasm is demonstrated by the deep purple color. The neuropil is lightly and uniformly reactive for the enzyme. × 400.

Fig. 14 DPN diaphorase in the neurons of the corresponding nucleus in the brain of the young in protein deficiency. Although fewer neurons were seen in each section through the nucleus, the neuronal enzyme reactivity resembled that of normal brain. The reactivity of the neuropil was less uniform than that of the control. × 400.

Fig. 15 Paravertebral ganglion of a normal newborn rat. Acid phosphatase activity is present in the perinuclear granules of the ganglion cells, and less intensely as a diffuse reaction in the cytoplasm. × 400.

Fig. 16 Paravertebral ganglion cells of the neonatal young in protein deficiency. No acid phosphatase reactive granules were present in the weakly reactive cytoplasm. × 400.

Fig. 17 The black reaction product indicating the presence of lactic dehydrogenase is localized in intensely reactive coarse granules in the brown fat cells of the control newborn rat. × 1000.

Fig. 18 The lactic dehydrogenase reactive granules present in brown fat cells of the rat in protein deficiency are finer and less intensely reactive than those in the control tissue. × 1000.

Fig. 19 Duodenum of the newborn control rat showing the reactivity of alkaline phosphatase. The strongly positive reaction of the surface epithelium of the villi is most intense at the apical portions of the villi and is characteristically absent from the basal crypt cells. The villi are uniform in size and show a compact arrangement. × 80.

Fig. 20 Duodenum of the newborn young of a protein-deficient rat. Alkaline phosphatase reactivity is limited to the surface epithelium on the apical surface of some of the villi which are irregularly shaped and reduced in number. × 80.

Fig. 21 Leucineaminopeptidase reactivity of the jejunal epithelium in the control newborn rat. The reaction product is localized in the surface epithelium of the villi and is not present in the cells lining the crypts. × 100.

Fig. 22 Lack of leucineaminopeptidase reactivity in the jejunal epithelium of newborn protein-deficient rat. The section shows villi of two neighboring loops of the jejunum whose muscle walls lie back to back. The number of villi is reduced. × 100.

Fig. 23 Nonspecific esterase in jejunal epithelium of normal newborn rat. The blue reaction product is most intense on the luminal surface of the enterocytes, but is present throughout the cytoplasm of both crypt and villus epithelium. The clear cells within the epithelium are goblet cells. × 100.

Fig. 24 Jejunal epithelium of the newborn intestine in protein restriction. This tissue was incubated four times as long as that shown in the preceding figure in order to obtain any visible reaction. Since the response was extremely weak, this tissue was not counterstained. The difference in size of the villi in control and low protein tissues is apparent. × 100.

Fig. 25 Villi of normal neonatal intestine showing reactivity indicative of succinic dehydrogenase. This enzyme activity is localized in the supra- and infranuclear mitochondria of the enterocytes. × 100.

Fig. 26 Villi of the newborn intestine in protein deficiency showing reduced succinic dehydrogenase activity. × 100.

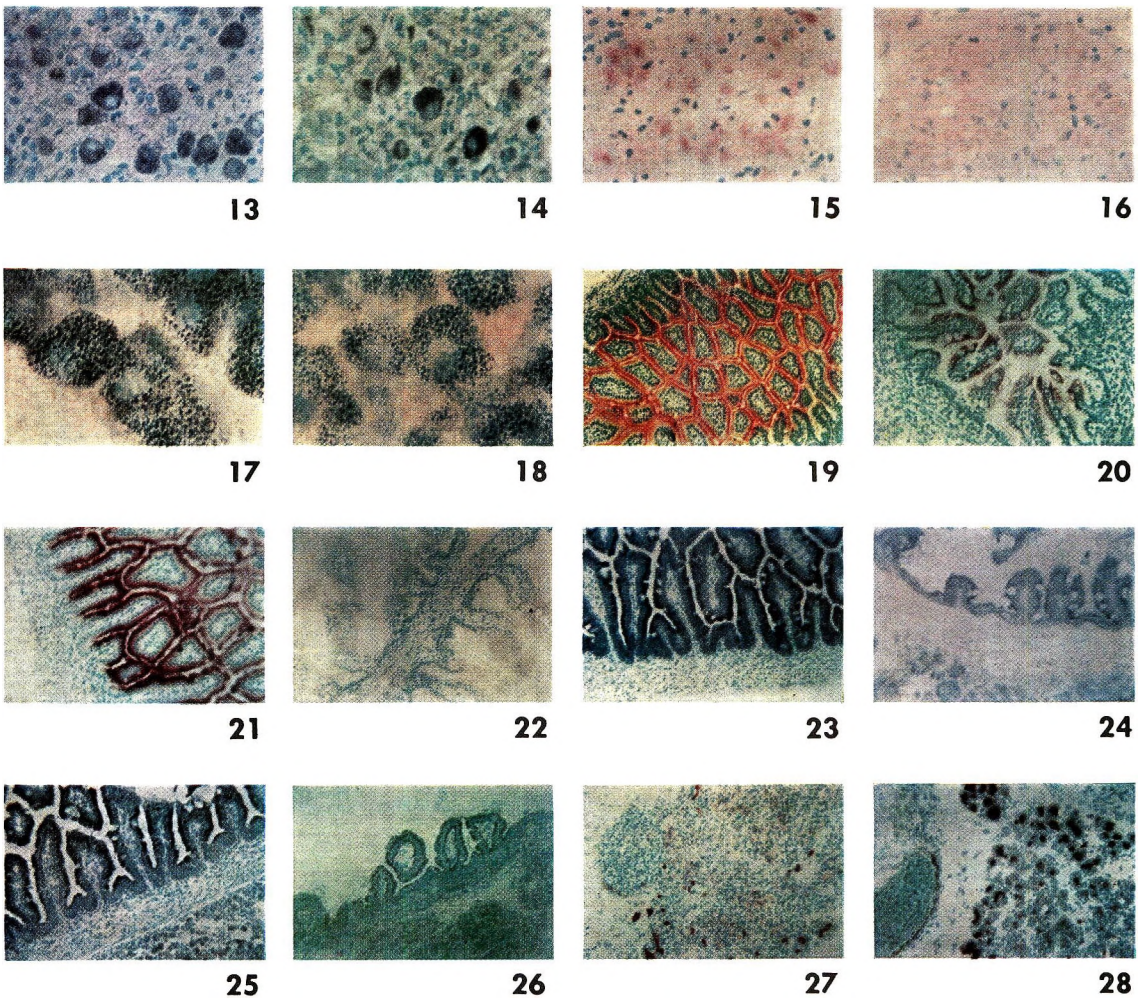
Fig. 27 Alkaline phosphatase reactivity in the thyroid of the control newborn rat. Only a few peripherally located follicles normally retain demonstrable alkaline phosphatase activity at birth. × 100.

Fig. 28 Thyroid tissue of the neonatal young of a protein-deficient rat showing the large number of intensely reactive follicles still present in this gland at birth. × 100.

denal epithelial cells. The more distal portions of the intestinal tract of low protein animals showed even greater reductions in enzyme activity (table 1). The jejunal epithelium contained no LAP (figs. 21 and 22), γ-GTP, or NS Est. activity (figs. 23 and 24) and drastically diminished concentrations of dehydrogenase and diaphorase reaction product (figs. 25 and 26). The epithelium of the ileum and colon were virtually devoid of their customary enzyme components (table 1). In all portions of the intestine, the characteristically occurring enzymes in the cells of the myenteric plexus reacted with normal intensity.

Liver: In addition to the changes in structure resulting from increased accumulation of lipids, hepatocytes of low protein newborn rats were found to contain reduced concentrations of Ac. P'ase, ATPase, SDH, MDH, LDH, G-6-PDH, DPN and TPN diaphorases, and Cytox. Of the enzymes normally present in newborn control liver tissue, MAO, NS Est. and LAP were absent.

Pancreas: The alkaline phosphatase activity of the acinar pancreas in low protein young was increased. This increase mimics the condition observed in 18- and 19-day fetal pancreatic tissue. All other enzymes normally present in control neonatal pan-



Figures 13 to 28

TABLE 1

Comparative intensity of enzyme reactivity in abnormal tissues of newborn low protein rats

Organs and tissues	Enzyme reactivity													
	Ac. P'ase	Alk. P'ase	ATPase	SDH	MDH	LDH	G-6-PDH	DPN Diaph	TPN Diaph	Cytox	MAO	NS Est.	LAP	γ-GTP
Intestine														
Duodenal epithelium	↓ [1]	↓	↓	↓	↓	↓	0	↓	↓	0	0	↓	0	0
Jejunal epithelium	0	0	0	0	↓	↓	0	0	0	0	0	↓	0	0
Ileal epithelium	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Myenteric plexus	=						=	=	=	=		=		
Liver														
Peripheral hepatocytes	↓		↓	↓	↓	↓	↓	↓	↓	↓	0	0	0	
Central hepatocytes	↓		↓	↓	↓	↓	↓	↓	↓	↓	0	0	0	
Nervous system														
Macronuclear neurons	=		=	=	=	=	=	=	=			=		
Purkinje cells	=		=	=	=	=	=	=	=			=		
Spinal cord neurons	=		=	=	=	=	=	=	=			=		
Paravertebral ganglia	↓		↓	↓	↓	↓	↓	↓	↓	0	0	0		
Choroid plexus	↓		↑	↓	↓	↓	0	↓	↓					
Pancreas														
Acinar cells	↓	↑	↓	0	↓	↓	0	↓	↓		0	↓		
Thyroid														
Follicle cells, peripheral follicles	=	↑	=	↓	↓	↓	↓	↓	↓					
Follicle cells, central follicles	=	↑	=	↓	↓	↓	↓	↓	↓					
Skin														
Epidermal stratum granulosum	0		0	0	0	0	0	0	0					
Epidermal stratum germinativum	↓		↓	↓	↓	↓	↓	↓	↓					
Hair epithelium	↓		↓	↓	↓	↓	↓	↓	↓					
Hair papilla		=					↓	↓	↓					
Brown fat														
Cells		↑	↓	↓	↓	↓	↓	↓	↓	↓	↓			

[1] ↑: Increased; ↓: decreased; 0: absent; =: unchanged; blank: tissue not reactive.

creas were detectable in this tissue from low protein animals, but the intensity of reactivity was much reduced (table 1).

Submaxillary Gland: In this tissue, where only limited numbers of ductile structures were found to be present in low protein tissue at birth, no alterations in enzyme distribution or intensity of reactivity were observed in histochemical preparations.

Skin: In those animals in which the granular and cornified layers of the epidermis were missing at birth, the enzymatic reactivity of the remaining layers of epidermal cells was much reduced, although the enzyme distribution remained normal. The epithelial cells of the rudimentary hair anlagen characteristic of the skin of low protein newborn also showed diminished activity of the enzymes characteristic of these cells in normal animals. The connective tissue components of the forming papilla were found to have reduced reactivity of G-6-PDH, and DPN and TPN diaphorases, but no change in Alk. P'ase activity (table 1).

Thyroid: Although morphological changes were not detected in the thyroid tissue of newborn low protein rat pups, histochemically demonstrable enzyme activity was found to be altered in the follicle cells of these animals. In neonatal control young, alkaline phosphatase activity was confined to a few peripherally located follicles and was of moderate intensity. In the thyroid of low protein pups and of normal 18-, 19-, and 20-day old fetal rats, Alk. P'ase activity was extremely intense and involved a majority of the follicles present in the outer half of the gland (figs. 27 and 28). Ac. P'ase and ATPase activity resembled the levels present in control tissue. The oxidative enzymes, however, were present in decreased concentration in the follicle cells of the low protein neonatal thyroid.

DISCUSSION

Despite the hypothetical uniformity of genetic constitution of the female rats used in these experiments, it was apparent that among these animals were some whose metabolic patterns resulted in conservation of their own tissues at the expense of those of the fetus. In others, the metabolic economy of the maternal animal was not protected to the same extent. The basis of this biological variability has not been determined, but it seems possible that even in highly inbred strains some degree of genetic segregation has occurred, producing a number of metabolic subtypes. The heterogeneity of physiological response shown by inbred animals has been extensively discussed in the genetic literature and has recently been reviewed by Lerner (14).

The alterations produced in the organs and tissues of young rats from the group most affected by maternal protein restriction were alike in degree and kind. The organs and tissues affected appeared to be those in which a considerable portion of growth and differentiation occurs during the last four days of gestation (15–18). As demonstrated by studies of DNA, protein, and weight relationships (1), cell production has been reduced during fetal development in some of the organs and tissues of these protein-deprived young. These findings have been corroborated and extended by the morphological observations made in the present portion of the investigation.

The hypoplasia resulting from decreased cell production has not been found to occur to the same extent in all affected organs. To some degree, the extent of the defect produced is apparently dependent on the kind and amount of developmental change which has yet to occur in the tissue. This is illustrated by the comparative effect of protein deprivation on brain, intestine and submaxillary gland. In the brain, most of the morphogenic activity thought to occur during the last 4 days preceding birth involves cell migration and reorientation (19). The several cell nuclei which differentiate during this time, appear to contain reduced numbers of neurons. The thinning of the cellular layers of the cerebral cortex and the apparent decrease in Purkinje cells in the cerebellum may, to some extent, reflect migrational changes. It would appear that cell proliferation is also involved since measurement of total DNA (1) has shown that decreased numbers of cells are indeed present in these brains. The gross architecture of the brain is not, however, dramatically affected. Further studies, in

depth, of changes in the brain are currently underway.

The intestinal mucosa, on the other hand, shows marked effects of the imposed protein deficit. In this tissue the attainment of definitive structure involves the transformation of a relatively short tubular lining of stratified squamous epithelium to a greatly expanded covering for a complex system of crypts and villi containing highly specialized cells (15). The inhibition of both cell production and cell specialization which has been shown to occur in this tissue has resulted in the formation of a seriously deranged organ. The hypoplasia encountered in the submaxillary gland was seen to involve the development of the rudimentary duct structures which characteristically form at this time. Duct development occurs by extension and branching of the primordial tubule; retardation of this process has been shown to result in the formation of normally structured but fewer than normal ducts and terminal branches (20). Lack of evidence of cellular maturity of these tubules may simply reflect the fact that normally these cells do not contain highly specialized cell organelles at birth (21).

The organs in which the response to prenatal protein deprivation involved alterations in cell morphology all showed changes in enzyme activity which could be related to these abnormal cells. Tissues which showed retention of fetal patterns of enzyme activity, on the other hand, did not appear to be morphologically abnormal. In these tissues Alk. P'ase reactivity was elevated, whereas activities of the oxidative enzymes such as SDH, MDH, LDH, DPN diaphorase and Cytox were decreased. The existence of a possible relationship of high levels of Alk. P'ase to cellular differentiation has been previousy postulated (22, 23, 24). Histochemically demonstrable Alk. P'ase has been found to be present in high concentration in prenatal thyroid (22), intestinal enterocytes (18, 25), the ependyma of the brain (26), bronchiolar epithelium (27), and brown fat cells,[4] and to decrease in these cells around the time of birth. The morphological events which correspond to the periods of high or low Alk. P'ase activity have not, however, been specifically investigated. Since the Gomori method for the demonstration of Alk. P'ase can be used in conjunction with electron microscopy, it would be of considerable interest to examine this presumed relationship using these methods.

The occurrence of decreased concentrations of the oxidative enzymes in cells of low protein tissues presents a challenging problem for further study. Dehydrogenases, diaphorases and cytochrome oxidase have been shown to increase progressively in concentration during the normal course of development of brown fat cells,[5] cardiac muscle (28), intestinal enterocytes (18), epidermal epithelial cells (29), and the cells of the proximal convoluted tubules of the kidney (30). SDH, MDH, LDH, DPN diaphorase and Cytox have been identified histochemically and biochemically as having mitochondrial localization (31–33). Whether the reduced content of enzymes associated with mitochondria in tissues of low protein rats represents a reduction in the number of mitochondria present in these cells, or an abnormality of enzyme content within the mitochondrion is not known. Several observations and hypotheses have been reported to explain the reduction of mitochondrial enzyme content which has been found to occur in mutant yeast (34). These include the detection of isomeric forms of normally occurring enzymes, the hypothesis that membrane abnormalities may have given rise to abnormal binding sites for the intramitochondrial enzymes, thus blocking their activity, and the demonstration that failure of synthesis of a particular enzyme has occurred (32). The complexity of mammalian tissue makes it difficult to obtain mitochondria for chemical studies of enzymes from only one type of cell; the structure of the gland, the particular constitution of the mitochondria, and the magnitude of the variation of enzyme content may make brown fat of protein-deficient animals a uniquely useful tissue for biochemical investigation of alterations resulting from prenatal protein deprivation.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Laurence Burdick in preparation of diets

[4] Unpublished observations.
[5] Unpublished observations.

and in the breeding, care and feeding of animals, and of Margaret Manuel and Sylvia Veregge for assistance in histochemical procedures.

LITERATURE CITED

1. Zeman, F. J., and E. C. Stanbrough 1969 Effect of maternal protein deficiency on cellular development in the fetal rat. J. Nutr., *99:* 274.
2. Potter, E. L., and S. T. Thierstein 1943 Glomerular development in the kidney as an index of fetal maturity. J. Pediat., *22:* 195.
3. Hammond, J. 1961 Effect of nutrition on the stage of development of the young at birth in farm animals. In: Ciba Foundation Symposium on Somatic Stability in the Newly Born. Little, Brown and Company, Boston, p. 5.
4. Zeman, F. J. 1968 Effects of maternal protein restriction on the kidney of the newborn young of rats. J. Nutr., *94:* 111.
5. Hall, S. M., and F. J. Zeman 1968 Kidney function of the progeny of rats fed a low protein diet. J. Nutr., *95:* 49.
6. Kretchmer, N., R. W. Greenberg and F. Sereni 1963 Biochemical basis of immaturity. Annu. Rev. Med., *14:* 407.
7. Burstone, M. S. 1962 Enzyme Histochemistry and Its Application in the Study of Neoplasms. Academic Press, Inc., New York.
8. Wachstein, M., E. Meisel and A. Niedzwiedz 1960 Histochemical demonstration of mitochondrial adenosine triphosphatase with the lead-adenosine triphosphatase technique. J. Histochem. Cytochem., *8:* 387.
9. Pearse, A. G. E. 1961 Histochemistry Theoretical and Applied, ed. 2. Little, Brown and Co., Boston.
10. Barka, T., and P. J. Anderson 1963 Histochemistry: Theory, Practice and Bibliography. Hoeber Medical Division: Harper and Row, New York.
11. Glenner, G. G., H. J. Burtner and G. W. Brown, Jr. 1957 The histochemical demonstration of monoamine oxidase activity by tetrazolium salts. J. Histochem. Cytochem., *5:* 591.
12. Nachlas, M. M., B. Monis, D. Rosenblatt and A. M. Seligman 1960 Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, L-leucyl-4-methoxy-2-naphthylamide. J. Biophys. Biochem. Cytol., *7:* 261.
13. Albert, Z., M. Orlowski and A. Szewczuk 1961 Histochemical demonstration of gammaglutamyl transpeptidase. Nature, *191:* 797.
14. Lerner, I. M. 1954 Genetic Homeostasis. John Wiley and Sons, Inc., New York.
15. Kammeraad, A. 1942 The development of the gastro-intestinal tract of the rat. I. Histogenesis of the epithelium of the stomach, small intestine and pancreas. J. Morph., *70:* 323.
16. Sidman, R. L. 1956 Histogenesis of brown adipose tissue in vivo and in organ culture. Anat. Rec., *28:* 581.
17. Carpenter, E. 1959 Development of fetal rat thyroid with special reference to uptake of radioactive iodine. J. Exp. Zool., *142:* 247.
18. Vollrath, L. 1969 Über die Entwicklung des Dünndarms der Ratte. Morphologische, histochemische und experimentelle Untersuchungen. Ergebn. Anat. Entwickl.-Gesch., *41:* Heft 3.
19. Berry, M., A. W. Rogers and J. T. Eayrs 1964 Pattern of cell migration during cortical histogenesis. Nature, *203:* 591.
20. Bloom, W., and D. W. Fawcett 1952 Textbook of Histology. W. B. Saunders Company, Philadelphia.
21. Olivecrona, H., and N. Hillarp 1949 Studies on the submicroscopic structure of the epithelial cells of the intestine, pancreas, and kidney of rats during histogenesis. Acta Anat., *8:* 281.
22. McAlpine, R. J. 1951 Alkaline glycerophosphatase in the developing thyroid, pituitary, and parathyroid of the albino rat. Anat. Rec., *109:* 323.
23. Moog, F. 1953 The functional differentiation of the small intestine. III. The influence of the pituitary-adrenal system on the differentiation of phosphatase in the duodenum of the suckling mouse. J. Exp. Zool., *124:* 329.
24. Rogers, K. T. 1960 Studies on chick brain of biochemical differentiation related to morphological differentiation and onset of function. III. Histochemical localization of alkaline phosphatase. J. Exp. Zool., *145:* 49.
25. Masnerova, M., O. Koldovsky and K. Kubat 1966 Postnatal changes in phosphatase and nonspecific esterase activity in the large intestine of the rat. Experientia, *22:* 518.
26. Chiquone, A. D. 1954 Distribution of alkaline phosphatase monoesterase in the central nervous system of the mouse embryo. J. Comp. Neurol., *100:* 415.
27. Sorokin, S., H. A. Padykula and E. Herman 1959 Comparative histochemical patterns in developing mammalian lungs. Develop. Biol., *1:* 125.
28. Toth, A., and T. H. Schiebler 1967 Über die Entwicklung der Arbeits- und Erregungsleitungsmuskulatur der Herzens von Ratte und Meerschweinchen. Histologische, histochemische und electrophysiologische Untersuchungen. Z. f. Zellforsch., *76:* 543.
29. Hashimoto, K., K. Ogawa and W. F. Lever 1963 Histochemical studies on the skin. II. The activity of succinic, malic and lactic dehydrogenase systems during the embryonic development of the skin in the rat. J. Invest. Dermatol., *39:* 21.
30. Fisher, E. R., and J. Gruhn 1959 Maturation of succinic dehydrogenase and cytochrome oxidase in neonatal rat kidney. Proc. Soc. Exp. Biol. Med., *101:* 781.

31. Friede, R. L., and R. A. Pax 1961 Mitochondria and mitochondrial enzymes. A comparative study of localization in the cat's brain stem. Histochemie, 2: 186.
32. Lehninger, A. L. 1964 The Mitochondrion. Molecular Basis of Structure and Function. W. A. Benjamin, Inc., New York.
33. Scarpelli, D. G., and A. G. E. Pearse 1958 Physical and chemical protection of cell constituents and the precise localization of enzymes. J. Histochem. Cytochem., 6: 369.
34. Wagner, R. P. 1969 Genetics and phenogenetics of mitochondria. Science, 163: 1026.

Available Carbohydrate in Rapeseed Meal and Soybean Meal as Determined by a Chemical Method and a Chick Bioassay [1]

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ABSTRACT Available carbohydrate was determined using a chemical method and a chick bioassay. Results of the chemical assay showed that the total soluble sugars and starch in two samples of rapeseed meal and one sample of soybean meal were 15.0% and 23.6%, respectively. Results of the bioassay showed that the available carbohydrate in the same samples of rapeseed meal and soybean meal was 6.9% and 14.1%, respectively. The values obtained by bioassay are lower than those obtained by chemical analysis, which may be due to the fact that the bioassay reflects not only the amount of available carbohydrate but also its absorbability. Calculations indicate that the difference in amount of available carbohydrate in rapeseed meal and soybean meal accounts for 19% of the difference in metabolizable energy of these two feedstuffs.

The ability of the chick to utilize the energy in rapeseed meal is limited. Lodhi and co-workers (1) found the metabolizable energy content of rapeseed meal for the chick to be 1200 kcal/kg. This is approximately half the metabolizable energy content of soybean meal which according to Hill and Renner (2) is 2770 kcal/kg. In an attempt to explain why the energy of rapeseed meal is so low, studies have been conducted to determine the availability of its carbohydrate. For comparative purposes similar studies were conducted on soybean meal.

Up to the present, three general methods have been used for estimating the carbohydrate in foodstuffs which is available for metabolism. These include: 1) indirect estimation by difference, using the Weende chemical methods; 2) biological determination of digestibility coefficient in which the Weende chemical methods are used to analyze the feces and ingested food; 3) direct chemical determination of carbohydrates either singly or by class. Of these, digestibility coefficients should best indicate the carbohydrate available for metabolic purposes. Numerous investigators have found, however, that although cellulose and hemicellulose are not usually classified as metabolizable by monogastrics, 20 to 80% of the crude fiber and pentosans in the diet are not recovered in the feces. Thus, none of these methods estimate the carbohydrate which has actually been absorbed and is available for metabolism.

Recently, Renner and Elcombe (3), and Brambila and Hill (4) have shown that the growth of chicks fed diets in which soybean fatty acids served as the sole source of nonprotein energy is markedly increased by the addition of a dietary source of carbohydrates. This finding suggested that the available carbohydrate in rapeseed meal and soybean meal might be determined by comparing the growth response obtained when these meals served as the source of carbohydrate with the growth response obtained when the "carbohydrate-free" diet was supplemented with graded levels of glucose. Thus, the following studies were undertaken to develop a bioassay for determining the available carbohydrate in rapeseed and soybean meals. For comparative purposes, available carbohydrate was also determined using the chemical method of Clegg (5).

EXPERIMENTAL

The composition of the "carbohydrate-free" diet employed in the bioassay is shown in table 1. The diet was formulated to contain 15.4 kcal of metabolizable energy per gram protein, using the values

Received for publication June 2, 1969.
[1] Supported in part by a research grant from the National Research Council of Canada.

TABLE 1

Composition of "carbohydrate-free" diet [1]

Ingredients	Amount
	%
Soybean protein [2]	33.57
Glycine	0.93
DL-Methionine	1.19
Soybean oil	2.94
Vitamin mixture [3]	0.83
Mineral mixture [4]	8.07
Cellulose [5]	10.58
Soybean fatty acids [6]	41.41
Chromic oxide	0.44
Antioxidant [7]	0.037

[1] Calculated metabolizable energy content of diet = 5.14 kcal/g. Supplies 15.4 kcal/g protein.
[2] Promine, Central Soya, Chemurgy Division, Chicago 60639.
[3] Vitamin mixture supplied: (in mg/100 g of diet) thiamine, 1.47; riboflavin, 1.47; calcium pantothenate, 5.89; biotin, 0.06; pyridoxine, 2.94; niacin, 11.77; folacin, 0.44; menadione, 0.44; vitamin B_{12}, 0.007; choline chloride, 442; aureomycin, 1.47; and vitamin A, 1472 USP units; vitamin D_3, 221 ICU; vitamin E, 4.9 IU.
[4] Mineral mixture supplied: (in mg/100 g of diet) $CaHPO_4$, 3164; $CaCO_3$, 2193; NaCl, 883; KH_2PO_4, 1369; $MgSO_4$, 356; KI, 0.43; $FeSO_4 \cdot 7H_2O$, 40.91; $CuSO_4 \cdot 5H_2O$, 2.30; $ZnCO_3$, 16.92; $CoCl_2 \cdot 6H_2O$, 0.25; $NaMoO_4 \cdot 2H_2O$, 1.22; Na_2SeO_3, 0.032; and $MnSO_4 \cdot H_2O$, 48.6.
[5] Solka Floc, S.W.-40-A, Brown Forest Products, Limited, Montreal, Quebec.
[6] Prepared from soybean oil (Renner and Elcombe (3)).
[7] Ethoxyquin.

3.83 [2] and 8.65 kcal/g (6) for the metabolizable energy of soybean protein and soybean fatty acids, respectively. At this ratio, protein is present in sufficient quantities to promote rapid growth but is not in excess (6,7). Diets containing glucose in amounts to supply 0.018, 0.035, 0.070 and 0.105 g/g soybean fatty acids were formulated from the "carbohydrate-free" diet by replacing the energy in soybean fatty acids by an equicaloric amount of a mixture of glucose and soybean fatty acids. Diets containing rapeseed meal or soybean meal as sources of carbohydrate were maintained isonitrogenous by reducing the amount of soybean protein (Promine) in the "carbohydrate-free" diet, and isocaloric by adjusting the levels of soybean fatty acids in the diets. The levels of rapeseed meal and soybean meal used in the assay were calculated to contain less than the chick's requirement for carbohydrate which is approximately 0.05 g glucose/g soybean fatty acids. The metabolizable energy values of glucose, rapeseed meal and soybean meal used in formulating the diets were 3.64 (8), 1.20 (1), and 2.77 (2) kcal/g, respectively.

Male crossbred chicks (Dominant White × White Plymouth Rock) were fed, during a pre-experimental period of 1 week, a "carbohydrate-free" diet in which nonprotein energy was supplied by soybean oil. This diet was formulated from the "carbohydrate-free" diet (table 1) by replacing 41.41 parts soybean fatty acids by 38.68 parts soybean oil. At the end of this period, duplicate groups of 10 chicks were allocated to each treatment on the basis of body weight and rate of gain using McKittrick's method (9). The chicks were housed in electrically heated, thermostatically controlled battery brooders with raised wire screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum. The chicks were weighed weekly and feed wastage was determined daily.

Standard response curves for glucose were obtained by plotting total weight gained by chicks during the 2-week experimental period against the $\log_{10}$ (1 + g glucose/g soybean fatty acids). This response curve was used to calculate the carbohydrate contents of rapeseed meal and soybean meal from data on weight gain.

Chemical assays for available carbohydrate in rapeseed meal and soybean meal were conducted using the method of Clegg (5). In this method starch and soluble sugars were extracted and measured colorimetrically after the addition of anthrone. For comparative purposes nitrogen-free extract was also determined. AOAC methods were used for determining the proximate composition.

The proximate composition of the rapeseed meals used in this study has been reported previously (1). The soybean meal used contained 53.6% protein, 1.1% fat, 3.6% crude fiber, 6.5% ash and 35.2% nitrogen-free extract, moisture-free basis.

RESULTS AND DISCUSSION

Standard growth response curves obtained by plotting weight gains against dietary glucose level expressed as $\log_{10}$ (1 + g glucose/g soybean fatty acids) are shown in figure 1. The metabolic requirement for carbohydrate determined by in-

[2] F. W. Hill, unpublished data.

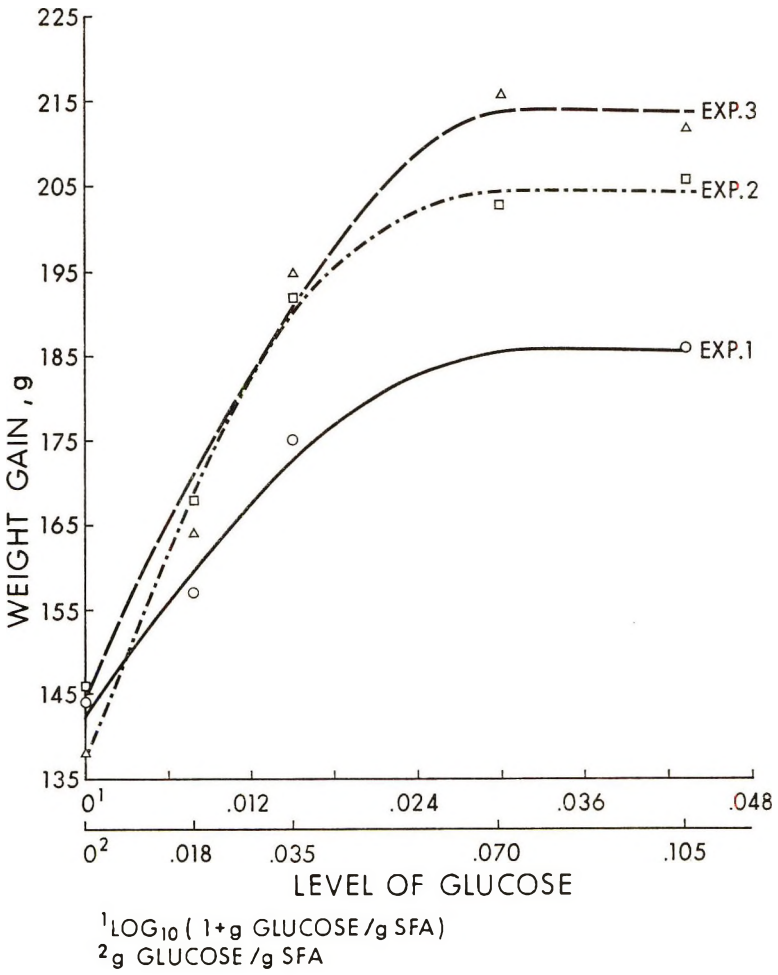

Fig. 1 Growth response of chicks fed graded levels of glucose expressed as $\log_{10}$ (1 + g glucose/g soybean fatty acids).

tersection of linear and plateau lines varied from 0.042 to 0.054 g glucose/g soybean fatty acids. Since the metabolic requirement for carbohydrate can be met from a wide variety of precursors, the requirement stated in the present study is only applicable under these conditions. These results confirm the finding of Renner and Elcombe (3) that the requirement for maximum growth response is in the range of 0.035 to 0.105 g glucose/g fatty acids.

Analysis of variance and application of Duncan's multiple range test (10) to the data on growth showed that in all experiments the addition of glucose up to a level of 0.070 g glucose/g fatty acids resulted in progressive and significant increases in growth. Supplementation with higher levels of glucose failed to cause additional growth response. Highly significant positive correlation coefficients (0.88, 0.88, 0.85) were obtained between level of carbohydrate in the three experimental diets and weight gain. Since growth was proportional to the level of glucose in the diet and since in previous studies [3] it was shown that fructose, galactose, xylose, sorbitol, dextrin and starch were as effective as glucose in stimulating growth of chicks

[3] Renner, R. 1966 Studies on the effectiveness of different carbohydrates in promoting the utilization of "carbohydrate- and glycerol-free diets." Federation Proc., 25: 303 (abstract).

fed "carbohydrate-free" diets, it was concluded that growth response could be used to determine the available carbohydrate in rapeseed meal and soybean meal even though carbohydrates other than glucose are present in rapeseed and soybean meals.

Data showing the available carbohydrate in rapeseed meal and soybean meal determined by the chick bioassay are summarized in table 2. Each meal was assayed at two or three levels, and each level was calculated to provide less than 0.05 g carbohydrate per gram soybean fatty acids. The data show that the available carbohydrate in rapeseed meal #5 was 4.5% and 6.3% in experiments 2 and 3, respectively, and for rapeseed meal #8 was 8.4% (experiment 3). Values for soybean meal obtained in experiments 1 and 2 were 14.0% and 14.2%, respectively. The variability in available carbohydrate observed within a given experiment is within the variation of biological experimentation.

Values for chemically available carbohydrate in nine samples of rapeseed meal and one sample of soybean meal determined by the method of Clegg (5) are summarized in table 3. For comparative purposes values for nitrogen-free extract are also given. Calculations indicate that in rapeseed meal soluble sugars and starch make up about 40% of the nitrogen-free extract, whereas in soybean meal soluble sugars and starch comprise 67% of the nitrogen-free extract.

Comparison of the data summarized in tables 2 and 3 shows that values for available carbohydrate obtained by Clegg's chemical method are higher than those obtained by the chick bioassay. This may be due to the presence in the extract of reducing compounds other than carbohydrate which react with the anthrone reagent. On the other hand, the difference could be due to the fact that the bioassay reflects not only the amount of these components but also their digestibility. Furthermore, the bioassay would also underestimate available carbohydrate if any of the carbohydrates were converted to volatile fatty acids through fermentation in either the crop or cecum. The extent to which

TABLE 2

Available carbohydrate in rapeseed meal and soybean meal determined using the chick bioassay

Level and source of CHO	Exp. 1		Exp. 2		Exp. 3	
	Avg wt gain [1]	CHO [2]	Avg wt gain [1]	CHO [2]	Avg wt gain [1]	CHO [2]
g/g SFA	*g*	*%*	*g*	*%*	*g*	*%*
Soybean meal						
0.143	—	—	177	16.1	—	—
0.175	162 [3]	13.0	—	—	—	—
0.214	—	—	187	14.5	—	—
0.286	—	—	189	11.9	—	—
0.350	189	15.1	—	—	—	—
Average	—	14.0	—	14.2	—	—
Rapeseed meal #5 [4]						
0.250	—	—	161	4.8	—	—
0.350	—	—	—	—	175	6.6
0.375	—	—	168	4.5	—	—
0.437	—	—	—	—	178	5.9
0.500	—	—	174	4.4	—	—
Average	—	—	—	4.5	—	6.3
Rapeseed meal #8 [5]						
0.375	—	—	—	—	183	8.3
0.500	—	—	—	—	195	8.5
Average	—	—	—	—	—	8.4

[1] From 7 to 21 days of age.
[2] Available carbohydrate.
[3] Values are averages of duplicate groups of 10 chicks.
[4] Solvent processed.
[5] Prepress-solvent processed.

TABLE 3

Carbohydrate content of soybean and rapeseed meals determined using Clegg's chemical method (5) [1]

	Nitrogen-free extract	Soluble sugars	Starch [2]	Available CHO [3]
	%	%	%	%
Soybean meal	35.3	18.6	5.0	23.6
Rapeseed meal #1	34.3	11.5	2.3	13.8
Rapeseed meal #2	36.6	10.8	3.3	14.1
Rapeseed meal #3	33.9	11.0	2.2	13.2
Rapeseed meal #4	36.1	12.4	2.2	14.6
Rapeseed meal #5	34.2	12.0	2.4	14.4
Rapeseed meal #6	35.2	11.2	2.2	13.4
Rapeseed meal #7	34.2	11.5	2.1	13.6
Rapeseed meal #8	35.3	12.4	3.2	15.6
Rapeseed meal #9	36.1	11.8	2.7	14.5
Average [4]	35.1	11.6	2.5	14.1

[1] Values are expressed on a dry-matter basis.
[2] Expressed as soluble sugars.
[3] Soluble sugars plus starch.
[4] Average of nine samples of rapeseed meal.

carbohydrates are fermented in the chick is unknown but may be insignificant because of the relatively rapid rate of passage of food through its gastrointestinal tract.

Bolton (11), from results of digestibility trials conducted with adult chicks, concluded that the sugars and starch in soybean meal determined chemically were 100% digestible. He reported the level of sugars and starch in soybean meal containing 49% protein on a dry-matter basis to be 17.6%. Subsequently, using an in vitro enzymatic method, Bolton (12) found the available carbohydrate in soybean meal (44% protein) to be 12.6% on a dry-matter basis. These results are in general agreement with results obtained in the present study using the chick bioassay.

The finding that the level of available carbohydrate in rapeseed meal is lower than in soybean meal helps to explain why the metabolizable energy value of rapeseed meal is low. Calculations, using results of the chick bioassay and assuming an energy value of 3.75 kcal/g for the carbohydrates in the two meals, indicate that the difference in amount of available carbohydrate in rapeseed meal and soybean meal accounts for 290 kcal/kg of the total difference in metabolizable energy value (1570 kcal/kg) of these two feedstuffs. Thus, 19% of the difference in metabolizable energy of the rapeseed meal and soybean meal may be accounted for by the difference in amount of available carbohydrates.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. T. Hardin of the Department of Animal Science, University of Alberta, for his assistance in connection with the statistical analysis of the data. The technical assistance of Mrs. Ulla De Bruijn and Mr. Ronald Wong is gratefully acknowledged.

LITERATURE CITED

1. Lodhi, G. N., R. Renner and D. R. Clandinin 1969 Studies on the metabolizable energy of rapeseed meal for growing chickens and laying hens. Poultry Sci., *48:* 964.
2. Hill, F. W., and R. Renner 1960 The metabolizable energy of soybean oil meals, soybean millfeeds and soybean hulls for the growing chick. Poultry Sci., *39:* 579.
3. Renner, R., and A. M. Elcombe 1964 Factors affecting the utilization of "carbohydrate-free" diets by the chick. II. Level of glycerol. J. Nutr., *84:* 327.
4. Brambila, S., and F. W. Hill 1966 Comparison of neutral fat and free fatty acids in high lipid-low carbohydrate diets. J. Nutr., *88:* 84.
5. Clegg, K. M. 1956 Application of the anthrone reagent to the estimation of starch in cereals. J. Sci. Food Agr., *7:* 40.
6. Renner, R. 1964 Factors affecting the utilization of "carbohydrate-free" diets by the chick. I. Level of protein. J. Nutr., *84:* 322.
7. Renner, R., and A. M. Elcombe 1967 Protein as a carbohydrate precursor in the chick. J. Nutr., *93:* 25.

8. Anderson, D. L., F. W. Hill and R. Renner 1958 Studies of the metabolizable and productive energy of glucose for the growing chick. J. Nutr., *65:* 561.
9. McKittrick, D. S. 1947 The selection of chicks for growth experiments and the evaluation of growth. Growth, *11:* 89.
10. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics with Special Reference to the Biological Sciences. McGraw-Hill Book Company, New York
11. Bolton, W. 1957 The digestibility by adult fowls of wheat fine middlings, maize germ meal, maize gluten feed, soybean meal and ground nut meal. J. Sci. Food Agr., *8:* 132.
12. Bolton, W. 1960 The determination of digestible carbohydrates in poultry foods. Analyst, *85:* 189.

Retention of Radiocesium by Rats Before and After Weaning [1]

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ABSTRACT Rat pups were given intraperitoneal injections of ^{134}Cs at 4 days of age, and the retention of the radiocesium was followed by whole-body counting. Up to the time of weaning, the pups lost half of the radiocesium every 10.9 days. After weaning, the rate of loss increased markedly and the body retention function became multiexponential rather than the single exponential observed during nursing. Experimental procedures ruled out recycling of radiocesium through the dams and the physical nature of the diet after weaning as being contributory factors to the changes observed in cesium retention. The experiments suggest that the higher potassium intake after weaning may be responsible for the sharp change in retention.

In a preliminary study in this laboratory of the effect of age upon the retention of radiocesium by rats it was observed that suckling rats exhibited a greater retention of radiocesium than weaned rats weighing 50 or 100 g. It was also noted that at weaning the retention of cesium by these rat pups decreased sharply and was in accord with data obtained using weaned 50-g rats; moreover, the dam at the time of weaning contained as much or more radioactivity than did the average suckling young. Since it is characteristic of the rat to induce the nursing pup to urinate by licking its anal region, the results of the experiment suggested that the long retention time of radiocesium in the suckling could be due to recycling of radiocesium from dam to the pup. To investigate this possibility it was decided to repeat the experiments while attempting to keep the dam from having oral contact with the pups.

The experiments reported in this paper show that the rate of cesium excretion by the young rat was greater postweaning than preweaning and that recycling of radiocesium through the mother was insignificant. This suggested a dietary component must be involved. Since potassium is very likely to affect cesium metabolism (1), weanling rats were fed diets containing various potassium levels and the retention of a single dose of radiocesium was followed. The results of this experiment suggest that increased potassium intake after weaning may be responsible to a large degree for the increased excretion rate of radiocesium.

METHODS

Pregnant albino rats of the Sprague-Dawley strain [2] weighing between 250 and 300 g were used. For 3 days after the birth of the pups the female was allowed to nurse the young undisturbed. On day 4, in all but two cases, each pup in the litter was injected intraperitoneally with 0.05 ml of a solution of radiocesium that contained 95 μCi of ^{134}Cs in 50 ml of distilled water. Radioactivity of individual pups was counted within 1 hour of dosing and at least twice weekly thereafter in a small animal crystal scintillation counter [3] while confined in a disposable thin plastic container. The counts were corrected for increase in body mass, radioactive decay, and machine variation. The resulting retention curves were normalized so that all pups started with the same initial number of counts of ^{134}Cs at time zero.

After the rat pups were dosed with radiocesium the mother was separated from the young each day during the period from 0800 to 1700 hours. At 1700 hours the mother was fitted with a fine wire screen head mask and returned to the cage con-

Received for publication July 15, 1969.

[1] Supported in part by the U.S. Atomic Energy Commission under contract AT(30-1)-2147.

[2] Blue Spruce Farms, Altamount, N. Y.

[3] Tobor, Nuclear-Chicago Corporation, Chicago, Ill.

taining her pups. The dam was provided with food and water only when she was caged separately from the pups. This procedure prevented oral contact and, moreover, served to control the intake of foods other than maternal milk by the pups.

To demonstrate the effect of weaning upon the retention of radiocesium, rat pups were weaned at 16 days, 3 weeks, or 4 weeks of age. In most cases the pups were fed normal pelleted rat diet [4] during the absence of and after final separation from the dam. Three litters of pups, however, were provided with fresh cow's milk instead of the solid diet. The experimental variables of this phase of the research are presented in table 1.

These experiments suggested that weaning, per se, was not the causative factor but that some component of the diet might be altered at weaning, increasing the excretion of cesium. A comparison of the diets suggested potassium as being the responsible agent.

To test the effect of dietary potassium, male weanling rats were each given 0.2 ml of the radiocesium dosing solution intraperitoneally and then placed on purified diets containing various levels of potassium. The basic diet was essentially a sucrose, casein, corn oil mix to which were added minerals and vitamins to supply all the known requirements of the rat.[5] Potassium levels were 0.11, 0.22, 0.44 and 0.88%, respectively, for the four groups.

RESULTS

Figure 1 is a graphic presentation of the results of the series of trials in which time of weaning was varied in order to determine the degree of association of weaning to the abrupt change in radiocesium rentention. In drawing the curves, averages of litter values at each sampling point were used rather than averages weighted for the number of animals in a litter. During the period the pups were nursing the retention of radiocesium was greatly different than for the period after weaning; moreover, the sharp break in the retention curve came at or closely after the time of weaning. The retention of radiocesium in counts per minute by all nursing pups could be described by the single exponential function:

$$\text{cpm} = 2.75 \times 10^5 \, e^{-0.064t}$$

This represents a half-time for radiocesium retention of 10.9 days.

After weaning, the falloff of the retention curve could be expressed only as the sum of two or more exponential functions; the values of the parameters in the functions for the postweaning period for the various experimental groups are shown in table 2. Note that the time of weaning has been used as day zero, and that the relative sizes of the compartments (a_1, a_2, a_3) are expressed as a percentage of the initial number of counts. The first three groups in table 2 are those depicted in figure 1. Of importance is the observation that the k values for any one compartment were almost identical for the various groups. The half-times were about 3.0 and 8.7 days for k_1 and k_2, respectively, and when compared to the half-time for the preweaning period

[4] Big Red Laboratory Diet, Agway, Inc., Syracuse, N. Y. This diet contained 0.76% potassium.

[5] Each kilogram of diet contained: (in grams) sucrose, 680; vitamin-free casein, 180; corn oil, 80; brewer's dried yeast, 20; vitamin mix (Nutritional Biochemicals), 20; and salt mix, 24. The salt mixture contained: (in grams) $Ca(H_2PO_4)_2 \cdot H_2O$, 320.85; $CaHPO_4$, 173.4; $FeCl_3 \cdot 6H_2O$, 36.0; $NaH_2PO_4 \cdot H_2O$, 115.65; and NaCl, 65.25. To achieve the various potassium levels, either 2.76, 5.53, 11.05 or 22.05 g potassium were added to each kilogram of diet.

TABLE 1

Protocol for management of rats during the series of experiments relating weaning and diet to cesium retention

Supplemental diet provided pups	Age at weaning	Number of litters	Number of pups in litter	Age of pups at dosing
Pelleted rat diet	16 days	2	6, 6	4 days
Pelleted rat diet	3 weeks	1	10	7 days
Pelleted rat diet	3 weeks	1	3	4 days
Pelleted rat diet	4 weeks	2	7, 5	5 days
Pelleted rat diet	4 weeks	1	6	4 days
Fresh cow's milk	4 weeks	3	5, 5, 6	4 days

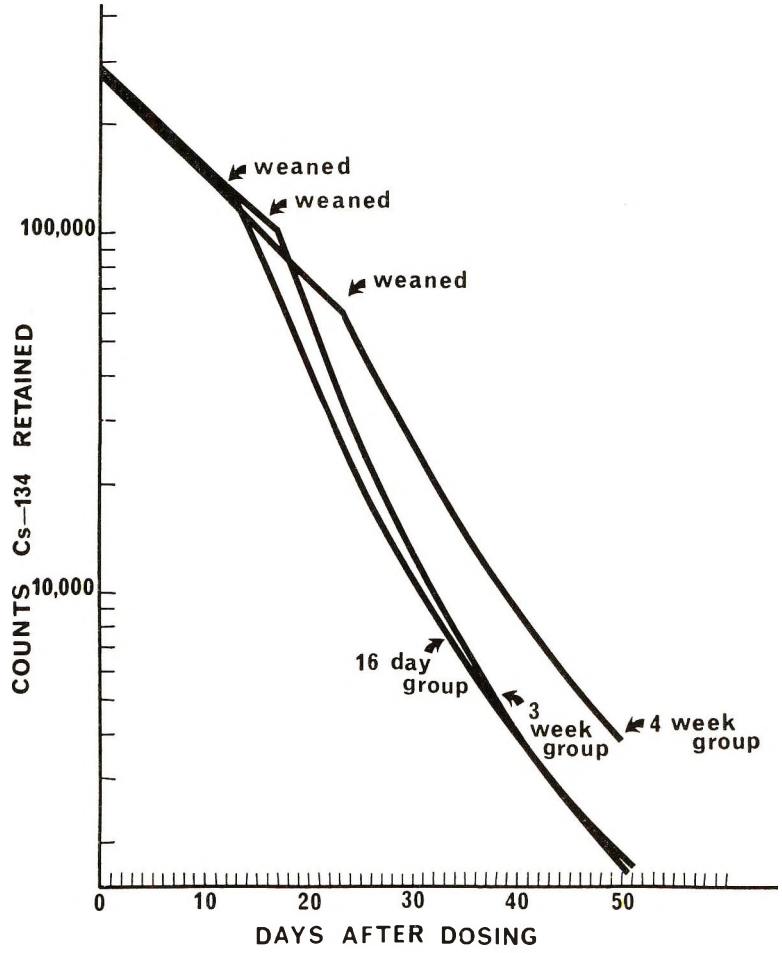

Fig. 1 Retention of a single intraperitoneal dose of radiocesium for 3 values of the time of weaning.

TABLE 2

Values of the parameters in the exponential functions describing the retention of radiocesium by young rats [1]

Groups	a_1	k_1	a_2	k_2	a_3	k_3
	%		%		%	
Weaned at 16 days [2]	77.8	0.23	22.3	0.08		
Weaned at 3 weeks [2]	71.1	0.23	28.9	0.09		
Weaned at 4 weeks [2]	57.1	0.22	42.9	0.08		
Weaned at 4 weeks (milk diet)	47.4	0.27	52.6	0.08		
Weanling rats dosed at [2,3] 50 g body weight	15.4	1.04	72.5	0.25	12.1	0.08

[1] The exponential takes the form:

$$\text{Cesium retention} = a_1 e^{-k_1 t} + \ldots + a_n e^{-k_n t}.$$

[2] Animals received normal pelleted rat diet after weaning.

[3] This experiment lasted 50 days in contrast to the approximately 30 days on the test diet for the other groups.

demonstrate that the excretion of radiocesium was accelerated after weaning. The sizes of the compartments varied between the groups and, at least for the first three groups, can be explained to be an interaction between the rate of loss of radiocesium from the first compartment and the age at which weaning occurred.

When the pups of the various experimental groups were weaned the dams were killed and the radiocesium content of the whole animal and that of the pelt were determined. On the average a dam contained 13.24 ± 4.8% (mean ± SEM) of the radiocesium found in one pup of her litter at weaning. Of this count, 55 ± 11% was associated with the pelt. In contrast, in an experiment (three litters) where masks were not used the mother contained 124 ± 42% of the radiocesium found in an average pup.

Figure 2 presents the results of a trial in which the pups were given either the normal pelleted rat diet (grain) or fresh cow's milk as the sole food after weaning. Again the sharp break in cesium retention was observed to occur at weaning with the difference between groups, as judged by body cesium content at the end of the experiment, being not statistically significant. The k values for these two groups were also quite similar (table 2).

When weanling rats were fed purified diets at four different potassium levels it was found that the rate of cesium excretion was increased (fig. 3). The decrease in 31 days was about 56, 74, 85 and 94% for the 0.11, 0.22, 0.44, and 0.88% K diets, respectively. It is to be noted that rats fed the commercial rat diet (0.76% K) at weaning time showed a decrease in body radiocesium of about 94% in the 30 days after weaning (fig. 2).

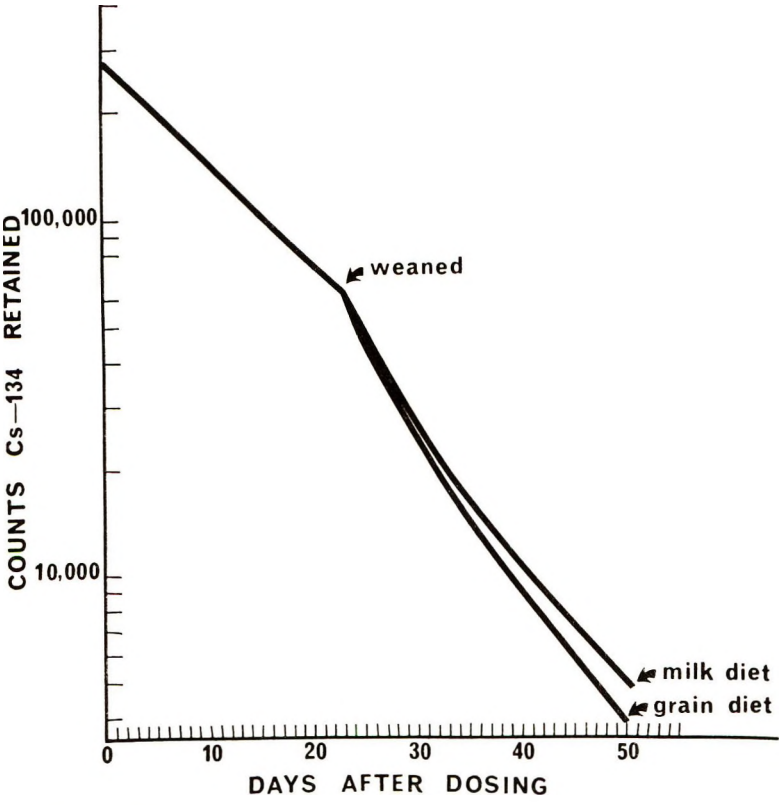

Fig. 2 Retention of radiocesium by rat pups weaned at 4 weeks of age to a diet of liquid cow's milk or a normal pelleted rat diet.

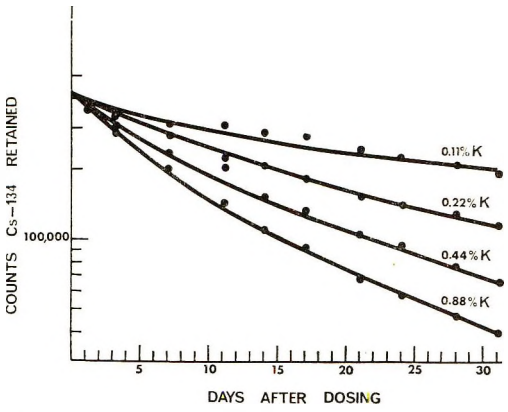

Fig. 3 Retention of a single intraperitoneal dose of radiocesium by weanling rats fed diets differing only in their potassium content.

DISCUSSION

The results of these experiments demonstrate that for the rat there is a sharp change in the retention of radiocesium that occurs precisely at weaning. These results are in accord with those obtained by Matsusaka and Inaba (2) using mice; however, the change in the cesium retention at weaning is far more abrupt in the experiments reported here. The ability of a single exponential equation to express the body content of radiocesium for rats during the suckling period is evidence that radiocesium excretion did not accelerate with age as was the case for the suckling mice.

The radiocesium content of the mothers shows that the use of masks was effective in preventing the recycling of radiocesium back to the pups through the milk and eliminates recycling as an explanation of the phenomenon.

The rate constants for radiocesium in a particular mathematical compartment (table 2) after weaning were greatly similar regardless of the time of weaning and were similar to the rate constants determined for the second and third components of the equation describing the cesium retention of rats given radiocesium after weaning. This suggests that the age of these animals did not contribute to the results.

Elimination of recycling and age of pups as explanations leaves only the difference between pre- and postweaning diets as the most probable factor. Stewart et al. (3) have presented evidence that the physical character of the diet can markedly alter the amount of ingested radiocesium secreted in cow's milk. A major change that usually occurs at weaning is the transition from an easily digested fluid diet to a diet high in fiber and other components with relatively limited digestibility. These substances within the digestive tract can create a physiological compartment that is much larger than that found in the milk-fed animal and can compete for cesium by chemical binding or chelation, and in so doing produce a greater excretion of radiocesium. The results of the experiment testing the commercial rat diet against fluid milk (fig. 2) showed no difference between groups, and one can only conclude that the fiber present in the pelleted diet (4.4%), in addition to the unknown amounts of natural chelators, made no significant contribution to the marked decrease in retention that began at weaning.

The provision of liquid cow's milk to the weanlings was also intended to continue the rat pups on a diet that was quite similar in composition to maternal milk. When rat's milk (4) and cow's milk (5) are compared directly it is observed that they differ greatly in composition. This is due mostly to the rat's milk having about 28% solids compared to about 12% for cow's milk. If, however, the comparison is made on the basis of amounts of the respective milks that will provide equicaloric intakes, the differences mostly disappear (table 3). From table 3 it is evident that, outside of the higher lactose and potassium of cow's milk, few differences existed in the nutrients supplied by equicaloric amounts of the milk of these two species.

The last column of table 3 shows the amount of each of these nutrients that would be available to the weanling rat when consuming an amount of the pelleted rat diet calorically equivalent to 100 g of rat milk. Lactose is not involved in the shortening of the radiocesium turnover times since there was no significant difference in the retention of radiocesium for weanling rats fed the pelleted diet or the fluid cow's milk (fig. 2). This leaves potassium as the most probable cause for the marked decrease in retention of cesium

TABLE 3

Various nutrients of rat's milk, cow's milk, and pelleted rat diet equated to a caloric intake equivalent to that contained in 100 g rat's milk

Nutrient	Rat's milk [1]	Cow's milk [2]	Pelleted rat diet
Calories	155	155	155
	g	*g*	*g*
Protein	8.9	7.3	10.5
Lactose	3.5	10.6	—
Fat	11.7	8.1	2.2
Ash	1.3	1.6	2.9
Ca [3]	0.24	0.27	0.54
Mg [3]	0.06	0.03	0.01
P	0.23	0.21	0.35
K [3]	0.11	0.31	0.33
Na	0.14	0.13	0.21

[1] Based upon analyses made by Luckey et al. (4).
[2] Adapted from values compiled by Macy and Kelly (5).
[3] Analysis of the milk of 7 rats in this laboratory by atomic absorption spectrophotometry indicated the milk of the dams contained 0.18% Ca, 0.06% Mg and 0.16% K. These values are in substantial agreement with Luckey et al. (4).

that occurs at weaning. This possibility was tested by feeding weanling rats diets containing different levels of potassium. The level of potassium markedly influenced cesium excretion, with the highest level (0.88% K) producing a retention of radiocesium similar to that of weanling rats fed the pelleted rat diet containing 0.76% K (fig. 3). The data thus suggest that during the suckling period the rat pup is receiving a diet relatively low in potassium, to which the pup responds by conserving potassium. Because of the chemical similarity to potassium the turnover time of cesium is also increased. When weaning occurs and the pup receives a diet relatively high in potassium the turnover time for the alkali metals is shortened, and the radiocesium is seen to decrease at a much faster rate than during the suckling period.

If extrapolation is made to the human, the data suggest that the human infant while nursing may attain higher body burdens of radiocesium than would be the case for somewhat older children. Human milk has approximately the same caloric value as cow's milk (75 vs. 69 kcal/100 ml); however, the potassium content is much lower (0.05% vs. 0.14%). Therefore, if the number of calories ingested controls the volume of milk ingested, infants fed only human milk would ingest only about one-third of the amount of potassium ingested by infants fed cow's milk.

LITERATURE CITED

1. Wasserman, R. H., and C. L. Comar 1961 The influence of dietary potassium on the retention of chronically ingested cesium-137 in the rat. Rad. Res., *15:* 70.
2. Matsusaka, N., and J. Inaba 1967 Whole body retention of caesium-137 in suckling and weanling mice. Nature, *214:* 303.
3. Stewart, H. F., G. M. Ward and J. E. Johnson 1965 Availability of fallout Cs^{137} to dairy cattle from different types of feed. J. Dairy Sci., *48:* 709.
4. Luckey, T. D., T. J. Mende and J. Pleasants 1954 The physical and chemical characterization of rat's milk. J. Nutr., *53:* 345.
5. Macy, I. C., and H. J. Kelly 1961 Human milk and cow's milk in infant nutrition. In: Milk: the Mammary Gland and Its Secretion, vol. II, eds., S. K. Kon and A. T. Cowie. Academic Press, New York, pp. 265–304.
6. Jonhson, J. E., D. Garner and G. M. Ward 1967 Influence of dietary potassium, rubidium, or sodium on the retention time of radiocesium in rats. Proc. Soc. Exp. Biol. Med., *217:* 857.

Zinc Deficiency and Incorporation of ^{14}C-labeled Methionine into Tissue Proteins in Rats

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ABSTRACT Studies to determine the role of zinc on the rate of incorporation of ^{14}C-labeled methionine into tissue proteins were undertaken. The results show that rats fed a semipurified diet deficient in zinc incorporated significantly less DL-methionine-2-^{14}C into plasma, liver, kidney and muscle at 2 hours postinjection than rats receiving a zinc-supplemented diet. Similar results at 4 and 8 hours were noted in liver and kidney. The same trends were also observed when DL-methionine-1-^{14}C or L-methionine-methyl-^{14}C was injected. When unlabeled methionine was injected, all three groups of zinc-deficient rats showed a significant decrease in the specific activities of liver and kidney protein as compared with zinc-supplemented rats. Conversely, the incorporation of all three ^{14}C-labeled methionines into pancreatic protein at 2 hours was significantly greater in zinc-deficient rats than in zinc-supplemented rats. This enhanced conversion disappeared when zinc-deficient animals were previously injected with nonradioactive methionine. Total radioactivity as well as radioactivity in TCA-soluble fractions of liver and kidney were unaffected by zinc deficiency; therefore, the reduction in protein synthesis was probably unrelated to methionine uptake. It was further demonstrated that the effects on the incorporation of labeled methionine into tissue proteins were due to zinc deficiency per se rather than to reduced food intake.

The discovery that zinc is a component in several enzymes (1) led to the speculation of a close relationship between enzyme activity and the abnormalities seen in zinc-deficient animals. Results from this laboratory (2) demonstrated that zinc deficiency in rats reduced the activity of pancreatic carboxypeptidase A, but had no significant effect on pancreatic carboxypeptidase B or liver alcohol dehydrogenase. Later Mills et al. (3) found that a reduction of pancreatic carboxypeptidase activity in zinc-deficient rats can be restored to normal on zinc therapy. Our further studies (4) indicated that the activities of kidney and tibia alkaline phosphatases and liver lactic dehydrogenase were greatly decreased in zinc-deficient rats. Similar decreases in enzyme activity were observed by Prasad et al. (5) when the activities of dehydrogenase and phosphatase were determined by histochemical methods. Kfoury and associates (6), however, reported that alcohol dehydrogenase activity in liver was less in zinc-deficient rats than in control animals. No difference was observed in alkaline phosphatase from kidneys of zinc-deficient and control animals. Swenerton and Hurley (7) showed that rats with severe deficiency of zinc still maintained the normal activities of liver lactic dehydrogenase and glutamic dehydrogenase. Although the reasons for the apparently inconsistent findings from various workers are not known, one thing seems to be clear: alterations in the activities of zinc metalloenzymes are not the major cause for the changes associated with zinc deficiency.

Studies by Nielsen et al. (8, 9) indicated that zinc deficiency symptoms in chicks could be partially alleviated by giving histidine or cysteine supplement. Zinc was also shown to be essential in the regulation of an important tripeptide, reduced glutathione (10). Recently we reported oxidation of L-methionine-methyl-^{14}C in zinc-deficient rats to be much faster than in zinc-supplemented animals (11). Increased oxidation of other amino acids by zinc-deficient rats has been reported by Theuer and Hoekstra (12). It seemed reasonable to suppose that observed defects in amino acid metabolism in zinc-deficient animals might be associated with alteration in the rate of protein synthesis. Indeed, stimulus for further investigation appeared in the observation

Received for publication May 14, 1969.

that zinc is required for protein synthesis in *Rhizopus nigricans* (13) and *Euglena gracilis* (14). It has also been shown that carbonic anhydrase activity in zinc-deficient tomato plants was restricted, not by a lack of sufficient zinc to activate an apoenzyme but by the blocking of metabolic reactions forming protein (15), and that the levels of free amino acids were increased (16).

The present investigation was conducted to determine the effect of zinc deficiency on the rate of incorporation in vivo of ^{14}C-labeled methionine into tissue proteins of the rat. Radioactive methionine was chosen for a number of reasons: 1)available evidence indicates that in pancreas this amino acid incorporated primarily into exocrine enzymes with the exception of one molecule found in one type of rat insulin (17); 2) the conversion of methionine-methyl-^{14}C to expired $^{14}CO_2$ appears to be much greater in zinc-deficient rats than in zinc-supplemented rats (11); and 3) cystine, a metabolic product of methionine, is one of the important amino acids in the composition of hair and skin (of interest since various lesions of the integument, including alopecia, eczema, thickening and keratinization of the epidermis, occurred during zinc deficiency).

EXPERIMENTAL

Animals and diets. Male rats of the Sprague-Dawley strain, 22 to 25 days old and weighing 45 to 60 g, were obtained commercially [1] and used exclusively. The rats were individually housed in stainless steel cages in a temperature-controlled laboratory. Zinc-supplemented diet was prepared by adding 80 to 90 mg of zinc as zinc carbonate to each kilogram of basal diet which contained less than 2 ppm of zinc. The basal diet, referred to as the zinc-deficient diet, contained: (in percent) sucrose, 65.97; dried egg white, 15:00; salt-free casein hydrolysate, 3:00; Mazola oil, 10:00; salt mixture, 5.74 [2]; and vitamin supplement, 0.29.[3]

Animals were fed ad libitum in all experiments except one in which pair-fed controls were used. The rats were allowed deionized, distilled water ad libitum. After 14 to 17 days the animals were studied as described below.

Injection of ^{14}C-labeled methione.[4] After overnight fasting, each rat was weighed and injected intramuscularly (1 μCi/100 g of body weight) with either DL-methionine-2-^{14}C, DL-methionine-1-^{14}C, or L-methionine-methyl-^{14}C. The actual quantity of solution injected was obtained by difference in syringe weight before and after injection. Rats were killed by decapitation at stated intervals after isotope administration. Liver, pancreas, kidneys and a portion of gastrocnemius muscle were excised. All tissues were immediately homogenized with ice-cold water to make a 5% homogenate. An equal volume of cold 10% trichloroacetic acid (TCA) was added to the homogenate. Plasma was separated from heparinized blood by centrifugation at 1500 rpm for 10 minutes. An aliquot of plasma was mixed with two volumes of 10% TCA. The TCA suspensions from various tissues and plasma were kept at 4° for 24 hours.

Preparation of ^{14}C-labeled protein. For the preparation of ^{14}C-labeled protein, the procedure of Wool and Krahl (18) was modified as follows: The 10% TCA suspension was centrifuged, and the sediment was washed thrice successively with 5 ml of 5% TCA containing 3.73 mg of unlabeled DL-methionine. The residue was then dissolved in 3 ml of 88% formic acid and 0.6 ml of 30% hydrogen peroxide, and the mixture allowed to stand 30 minutes at 25-28°. To the formic acid-treated solution, 5 ml of 10% TCA was added, the resulting precipitate collected by centrifugation, washed twice with acetone and allowed to dry for a few minutes at room temperature. The protein precipitates were digested by adding 1 ml of Hyamine [5] and heating in

[1] Purchased from Zivic Miller Laboratories, Inc., 3848 Hieber Road, Allison Park, Pa. 15101.

[2] Furnished per 100 g of diet: (in grams) $CaHPO_4$, 2.716; $CaCO_3$, 0.957; Na_2HPO_4, 0.670; NaCl, 0.383; KCl, 0.670; $MgSO_4$, 0.287; $FeC_6H_5O_7 \cdot 5H_2O$, 0.019; $MnSO_4$, 0.021; KIO_3, 0.001; and $CuSO_4 \cdot 5H_2O$, 0.001.

[3] Furnished per 100 g of diet: (in milligrams) thiamine·HCl, 0.24; riboflavin, 0.60; pyridoxine·HCl, 0.30; Ca pantothenate, 2.00; niacin, 4.00; inositol, 10.00; biotin, 0.20; folic acid, 0.20; vitamin B_{12}, 0.002; choline chloride, 100.00; 2-methyl-1,4-naphthoquinone, 1.00; and α-tocopherol, 6.00; (in IU) vitamin A, 2500; and vitamin D_3, 300.

[4] The following radiochemicals were obtained from New England Nuclear Corporation, Boston, Mass.: L-methionine-methyl-^{14}C (14.7 mCi/mmole), DL-methionine-2-^{14}C (2.33 mCi/mmole), and DL-methionine-1-^{14}C (4.15 mCi/mmole).

[5] Hyamine hydroxide, obtained from the Packard Instrument Company, Inc., Downers Grove, Ill.

a 45° water bath for 30 minutes or longer until completely dissolved.

In some experiments, a duplicate of 0.5 ml of 5% tissue homogenate was added to a counting vial containing 1 ml of Hyamine and the mixture heated at 55 to 60° for 1 hour or less until the tissue homogenate was completely digested. The TCA-soluble fractions obtained from liver or kidney homogenate were neutralized and the total volumes measured.

Radioisotope determination. The radioactivity in all samples was determined by mixing with the scintillation solution, diotol,[6] and counting in a Packard liquid scintillation spectrometer. Counting efficiency for radiocarbon was about 70%. An internal standard was used to correct for quenching. The results obtained from means of duplicate samples were further corrected for injected dose and the specific activity was expressed as counts per minute per milligram of protein as determined by the method of Lowry et al. (19) with bovine serum albumin as standard.

Statistical tests: The significance of the difference between the means of two groups of values was determined by Student's *t* test (20).

RESULTS

As reported elsewhere (10), growth was severely depressed by zinc deficiency (table 1 in reference 10). In those studies rats fed a low zinc diet for a 16-day experimental period gained weight at rates approximately 50% of rats fed an adequate level of zinc. In the present investigation the effect of zinc deficiency on the incorporation of DL-methionine-2-^{14}C into tissue proteins was studied at time intervals from 0.5 to 8 hours postinjection. The results in table 1 indicate that at 0.5 hour the specific activity present in selected tissues of zinc-deficient rats was approximately the same as in those of zinc-supplemented rats. At the end of 2 hours, significantly more DL-methionine-2-^{14}C was incorporated into pancreatic protein of rats fed diets low in zinc than in rats fed the diet containing an adequate level of zinc. On the other hand, zinc-deficient rats incorporated a smaller

[6] A mixture of 4.6 g 2,5-diphenyloxazole (PPO), 0.091 g 1,4-bis (2-(5-phenyloxazolyl-benzene)) (POPOP), 73 g naphthalene, 210 ml methanol, 350 ml dioxane, and 350 ml toluene.

TABLE 1

Incorporation of DL-*methionine-2-*^{14}*C into tissue proteins of zinc-supplemented and zinc-deficient rats at time intervals following injection*

Type of diet	No. of rats	Days on exp. diet	Hours after injection	Avg body weight	Specific activities				
					Pancreas	Plasma	Liver	Kidney	Muscle
				g	*cpm/mg protein*				
Zinc-supplemented	5	15	0.5	108 ± 17 [1]	167 ± 43	17 ± 3	97 ± 29	—	—
Zinc-deficient	5	15	0.5	71 ± 12	195 ± 25	18 ± 5	102 ± 14	—	—
Zinc-supplemented	6	14	2	112 ± 23	207 ± 55 [a]	139 ± 27 [b]	121 ± 16 [b]	299 ± 33 [a]	14 ± 4 [b]
Zinc-deficient	6	14	2	64 ± 11	389 ± 47	99 ± 24	99 ± 9	211 ± 26	8 ± 2
Zinc-supplemented	5	15	4	101 ± 5	305 ± 54	139 ± 16 [b]	132 ± 10 [b]	240 ± 18 [a]	—
Zinc-deficient	6	15	4	55 ± 4	301 ± 48	109 ± 8	109 ± 15	197 ± 23	—
Zinc-supplemented	6	16	8	123 ± 17	183 ± 26	156 ± 14 [b]	134 ± 12 [b]	312 ± 63 [b]	11 ± 2
Zinc-deficient	6	16	8	59 ± 4	169 ± 34	110 ± 38	112 ± 12	213 ± 59	8 ± 3

[1] Mean ± SD.
[a] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.01$).
[b] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.05$).

portion of injected DL-methionine-2-^{14}C into the plasma, liver, kidney and muscle proteins. At 4 and 8 hours postinjection, no significant differences were observed in the ^{14}C activity of pancreatic protein. There was again a significantly decreased incorporation of labeled methionine into liver and kidney proteins of zinc-deficient rats.

Seven experiments compared the effect of zinc deficiency on the incorporation of methionine-^{14}C labeled at carbon-1, carbon-2, and methyl-group positions. Results are given in table 2. With reference to the pancreatic protein fraction it was shown that the intraperitoneal injection of nonlabeled DL-methionine 10 minutes before the injection of methionine-^{14}C completely abolished the increased radioactivity in zinc-deficient rats, regardless of position of ^{14}C-labeling. When DL-methionine-2-^{14}C was injected, the amounts of radioactivity in TCA-insoluble fractions of the livers and kidneys of zinc-deficient rats were significantly less than those of zinc-supplemented animals. Similar trends were found between zinc-supplemented and zinc-deficient groups injected with DL-methionine-1-^{14}C or methyl-^{14}C although these amounts did not reach a statistical significance for liver in the case of the former, or for liver or kidney in the case of the latter. When nonlabeled methionine was injected, all 5 groups of zinc-deficient rats had a significant decrease in specific activities as compared with zinc-supplemented rats. These findings suggest an impairment of protein synthesis in rat liver and kidney during zinc deficiency.

To test whether the decreased ^{14}C-methionine incorporation was due to a general reduction in food consumption rather than to a specific effect of zinc deficiency, an experiment was performed using pair-fed zinc-supplemented rats. Data in table 3 show that rats receiving pair-fed zinc-supplemented diet incorporated significantly more DL-methionine-2-^{14}C into the plasma, liver and kidney than zinc-deficient rats. Conversely, in the pancreatic protein of the zinc-deficient rats, significantly more radioactivity was again observed.

The effect of zinc repletion on methionine incorporation into tissue proteins was determined by daily intraperitoneal injections of 400 μg of zinc (as zinc chloride) on the last 3 days of the experiment. Results summarized in table 4 indicated that liver protein in zinc-repleted rats compared with zinc-deficient rats had a significantly greater incorporation of DL-methionine-2-^{14}C after injection of nonlabeled methionine. In the kidney similar findings were noted except that the specific activity of zinc-repleted rats did not fully reach the value of zinc-supplemented rats. Thus, the defects in incorporation observed in zinc-deficient rats appeared to be readily reversible.

Results for the effect of zinc deficiency on the uptake of labeled methionine by the liver and kidney are given in table 5. The amount of the total radioactivity found in the whole liver and kidney, expressed as percentage of injected ^{14}C-labeled methionine, was about the same in zinc-deficient rats and zinc-supplemented rats. Similarly, no significant differences were observed in the radioactivity of TCA-soluble fractions. Values for the percentage of injected radioactivity incorporated into liver protein were significantly lower for zinc-deficient rats compared to zinc-supplemented rats, whether or not nonlabeled methionine was administered; in kidney protein the values do not differ significantly.

DISCUSSION

The biotin supplement reported here is less than that indicated by other authors (21) as effective for prevention of symptoms of biotin deficiency in an egg white diet containing inadequate zinc. They did not determine the minimum level needed under their conditions, however, and the reported effective level was probably in excess of that required. Increased oxidation of acetate-2-^{14}C to $^{14}CO_2$, a symptom of biotin deficiency (22), was not observed in rats fed our diet (11), and injection of biotin and other B-complex vitamins had no effect on growth or appearance.

Increased oxidation of L-methionine-methyl-^{14}C in zinc-deficient rats suggests a metabolic role of zinc in the regulation of methyl groups (11). The observations presented here indicate that zinc deficiency is associated with a reduction of ^{14}C-labeled methionine incorporation into liver and kidney proteins, but at present it is not

TABLE 2

Incorporation into tissue proteins of zinc-supplemented and zinc-deficient rats of ^{14}C-methionine labeled at various positions [1]

Type of diet	Compound used	No. of rats	Days on exp. diet	DL-Methionine (nonlabeled, 60 μmoles/100 g body wt)	Avg body wt	Specific activities		
						Pancreas	Liver	Kidney
					g	*cpm/mg protein*		
Zinc-supplemented	Methionine-1-^{14}C	5	17	+	135 ± 5 [2]	113 ± 16	54 ± 11 [b]	62 ± 5 [a]
Zinc-deficient	Methionine-1-^{14}C	6	17	+	65 ± 3	126 ± 35	38 ± 5	48 ± 5
Zinc-supplemented	Methionine-1-^{14}C	6	17	−	121 ± 3	249 ± 46 [b]	89 ± 13	230 ± 26 [b]
Zinc-deficient	Methionine-1-^{14}C	6	17	−	61 ± 1	308 ± 28	72 ± 13	182 ± 12
Zinc-supplemented	Methionine-2-^{14}C	6	14	+	108 ± 5	125 ± 18	77 ± 16 [b]	67 ± 8 [a]
Zinc-deficient	Methionine-2-^{14}C	6	14	+	55 ± 7	131 ± 26	59 ± 10	45 ± 5
Zinc-supplemented	Methionine-2-^{14}C	5	14	−	114 ± 9	225 ± 16 [a]	129 ± 10 [a]	232 ± 19 [b]
Zinc-deficient	Methionine-2-^{14}C	6	14	−	58 ± 4	376 ± 10	104 ± 12	197 ± 9
Zinc-supplemented	Methionine-methyl-^{14}C	6	15	+	115 ± 10	179 ± 21	132 ± 18 [a]	55 ± 9 [b]
Zinc-deficient	Methionine-methyl-^{14}C	6	15	+	56 ± 7	185 ± 16	63 ± 3	38 ± 5
Zinc-supplemented	Methionine-methyl-^{14}C	9	14	+ [3]	125 ± 15	—	87 ± 15 [a]	43 ± 5 [b]
Zinc-deficient	Methionine-methyl-^{14}C	8	14	+ [3]	63 ± 6	—	68 ± 7	38 ± 3
Zinc-supplemented	Methionine-methyl-^{14}C	6	16	−	120 ± 7	209 ± 66 [a]	71 ± 14	127 ± 26
Zinc-deficient	Methionine-methyl-^{14}C	6	16	−	62 ± 5	318 ± 67	68 ± 11	108 ± 11

[1] All rats killed 2 hours after isotope injection.
[2] Mean ± SD.
[3] Nonlabeled L-methionine used instead of DL-methionine.
[a] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.01$).
[b] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.05$).

TABLE 3

Incorporation of DL-*methionine-2-*^{14}*C into tissue proteins in pair-fed zinc-supplemented and zinc-deficient rats* [1]

Type of diet	Final body wt (14 days)	Specific activities			
		Pancreas	Plasma	Liver	Kidney
	g	*cpm/mg protein*			
Zinc-supplemented	84 ± 8 [2]	262 ± 19 [b]	122 ± 9 [a]	118 ± 11 [a]	243 ± 15 [a]
Zinc-deficient	57 ± 7	365 ± 88	70 ± 21	85 ± 8	176 ± 18

[1] All rats were killed 2 hours after isotope injection.
[2] Mean ± SD.
[a] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.01$).
[b] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.05$).

TABLE 4

Effect of zinc repletion on incorporation of DL-*methionine-2-*^{14}*C into tissue proteins* [1]

Type of diet	Final body wt (14 days)	Specific activities		
		Pancreas	Liver	Kidney
	g	*cpm/mg protein*		
Zinc-supplemented	104 ± 2 [2]	118 ± 23	66 ± 7 [a]	56 ± 3 [a,c]
Zinc-deficient	55 ± 5	94 ± 38	51 ± 3	39 ± 5
Zinc-deficient and -repleted [3]	60 ± 5	104 ± 20	60 ± 5 [b]	47 ± 5 [b]

[1] Six rats in each group were killed 2 hours after injection of DL-methionine-2-^{14}C. Ten minutes before isotope injection, nonlabeled DL-methionine was injected in the amount of 60 μmoles/100 g body wt.
[2] Mean ± SD.
[3] Each rat received an intraperitoneal injection of 400 μg zinc daily on last 3 days of experiment.
[a] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.01$).
[b] Difference between zinc-repleted and zinc-deficient rats is statistically significant ($P < 0.05$).
[c] Difference between zinc-supplemented and zinc-repleted rats is statistically significant ($P < 0.05$).

clear whether these are interrelated or are independent processes. If the decreased specific activities in the liver and kidney proteins of zinc-deficient rats are due to a decrease in the availability of precursors, a nonlabeled methionine supplement before the injection of radioactive methionine would be expected to correct the adverse effect. Experimental data (table 2) obtained by using such a procedure do not support this hypothesis. Also, in other studies [7] we have found that the specific activities of liver and kidney were significantly lower in zinc-deficient rats than in zinc-supplemented animals, 2 hours after DL-cystine-1-^{14}C injection. The latter findings suggested that zinc is involved in the utilization of a sulfur-containing amino acid other than that employed in the studies described here. The total radioactivity in the tissues examined was unaffected by zinc deficiency; therefore, the reduction in protein synthesis is probably unrelated to the uptake of methionine.

The pancreas, an organ having a very high metabolic activity, is known to synthesize more protein per gram of gland than any other body tissue (23). The present results confirm this in that the specific activity of the trichloroacetic-acid-insoluble fraction from the pancreas is much greater than that from the liver and kidney of the same rat.

The paradoxical effects of zinc deficiency on ^{14}C-methionine incorporation into the proteins of the pancreas, as opposed to the proteins of the liver and kidney, are of particular interest. The reasons for those phenomena, however, are not at all clear.

The enhancement of methionine incorporation into pancreatic protein of zinc-deficient rats does not appear to agree with the earlier reports of Mills et al. (3) indicating that protein synthesis in the pancreas of zinc-deficient rats is impaired as measured by the incorporation of ingested U-^{14}C-labeled *Chlorella* protein. This apparent disagreement might be partially explained by the difference in the labeled compounds used.

Our data show that the increased specific activity of pancreatic protein in zinc-deficient rats is abolished in animals previously treated with nonradioactive methio-

[7] Hsu, J. M., and W. L. Anthony, unpublished observations.

TABLE 5

Distribution of radioactivity in liver and kidney of zinc-supplemented and zinc-deficient rats [1]

Type of diet	DL-Methionine (nonlabeled, 60 μmoles/ 100 g body wt.)	Percentage of injected ^{14}C					
		Liver			Kidney		
		Total	TCA-soluble fraction	Protein	Total	TCA-soluble fraction	Protein
Zinc-supplemented	+	7.76 ± 0.47 [2]	2.85 ± 0.62	3.77 ± 0.14 [a]	1.22 ± 0.09	0.61 ± 0.03	0.49 ± 0.06
Zinc-deficient	+	7.32 ± 0.74	3.32 ± 0.59	3.14 ± 0.18	1.23 ± 0.18	0.74 ± 0.17	0.52 ± 0.13
Zinc-supplemented	−	9.16 ± 0.75	2.29 ± 0.42	6.41 ± 0.53 [b]	4.31 ± 0.69	1.24 ± 0.32	2.55 ± 0.30
Zinc-deficient	−	8.75 ± 0.71	1.97 ± 0.35	5.79 ± 0.33	4.33 ± 0.23	1.07 ± 0.01	2.52 ± 0.26

[1] Six rats in each group were killed 2 hours after DL-methionine-2-^{14}C injection.
[2] Mean ± SD.
[a] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.01$).
[b] Difference between zinc-supplemented and zinc-deficient rats is statistically significant ($P < 0.05$).

nine. This fact suggests a depletion of methionine. Whether this is also true for other amino acids has not been investigated. It seems apparent, however, that under the experimental conditions we have employed, the pancreatic cells (unlike the hepatic and renal cells), are still capable of synthesizing proteins whenever methionine becomes available. Thus, it is not surprising to find that the activities of several pancreatic exocrine enzymes—including lipase,[8] trypsin, and chymotrypsin (3), and carboxypeptidase B (2)—were unaffected by zinc deficiency. Nevertheless, available information does not rule out the possibility of an effect of zinc deficiency on the synthesis of specific proteins in rat pancreas.

LITERATURE CITED

1. Vallee, B. L. 1959 Biochemistry, physiology and pathology of zinc. Physiol. Rev., *39:* 443.
2. Hsu, J. M., J. K. Anilane and D. E. Scanlan 1966 Pancreatic carboxypeptidases: Activities in zinc-deficient rats. Science, *153:* 882.
3. Mills, C. F., J. Quarterman, R. B. Williams, A. C. Dalgarno and B. Panic 1967 The effects of zinc deficiency on pancreatic carboxypeptidase activity and protein digestion and absorption in the rat. Biochem. J., *102:* 712.
4. Hsu, J. M., and J. K. Anilane 1967 Effect of zinc deficiency on zinc metalloenzymes in rats. In: The Proceedings of the Seventh International Congress of Nutrition, vol. 5, eds., J. Kuehnsu and H. Diedrick Cremer, p. 753.
5. Prasad, A. S., D. Oberleas, P. Wolf and J. P. Horowitz 1967 Studies on zinc deficiency: Changes in trace elements and enzyme activities in tissues of zinc-deficient rats. J. Clin. Invest., *46:* 549.
6. Kfoury, G. A., J. G. Reinhold and S. J. Simonian 1968 Enzyme activities in tissues of zinc-deficient rats. J. Nutr., *95:* 102.
7. Swenerton, H., and L. S. Hurley 1968 Severe zinc deficiency in male and female rats. J. Nutr., *95:* 8.
8. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1968 Effect of some dietary synthetic and natural chelating agents on the zinc deficiency syndrome in the chick. J. Nutr., *89:* 35.
9. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1967 Effect of histamine, histidine and some related compounds on the zinc-deficient chick. Proc. Soc. Exp. Biol. Med., *124:* 1106.
10. Hsu, J. M., W. L. Anthony and P. J. Buchanan 1968 Incorporation of glycine-1-

[8] See footnote 7.

^{14}C into liver glutathione in zinc-deficient rats. Proc. Soc. Exp. Biol. Med., *127:* 1048.

11. Hsu, J. M., W. L. Anthony and P. J. Buchanan 1969 Zinc deficiency and oxidation of L-methionine-methyl-^{14}C in rats. J. Nutr., *97:* 279.
12. Theuer, R. C., and W. G. Hoekstra 1966 Oxidation of ^{14}C-labeled carbohydrate, fat and amino acid substrates by zinc deficient rats. J. Nutr., *89:* 448.
13. Wegner, W. S., and A. H. Romano 1963 Zinc stimulation of RNA and protein synthesis in *Rhizopus nigricans*. Science, *142:* 1669.
14. Wacker, W. E. C. 1962 Nucleic acids and metals. III. Changes in nucleic acid, protein, and metal content as a consequence of zinc deficiency in *Euglena gracilis*. Biochemistry, *1:* 859.
15. Wood, J. G., and P. M. Sibly 1952 Carbonic anhydrase activity in plants in relation to zinc content. Aust. J. Sci. Res. (Ser. B), *5:* 244.
16. Possingham, J. V. 1956 The effect of mineral nutrition on the content of free amino acids and amides in tomato plants. I. A comparison of the effect of deficiencies of copper, zinc, manganese, iron, and molybdenum. Aust. J. Biol. Sci., *9:* 539.
17. Klostermeyer, H., and R. E. Humbel 1966 The chemistry and biochemistry of insulin. Angew. Chem. Int. Ed., *5:* 807.
18. Wool, I. G., and M. E. Krahl 1959 Incorporation of ^{14}C-amino acids into protein of isolated diaphragms: An effect of insulin independent of glucose entry. Amer. J. Physiol., *196:* 961.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
20. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames, Iowa, p. 65.
21. Luecke, R. W., M. E. Olman and B. V. Baltzer 1968 Zinc deficiency in the rat: Effect on serum and intestinal alkaline phosphatase activities. J. Nutr., *94:* 344.
22. Gram, M. R., and R. Okey 1958 Incorporation of acetate-2-^{14}C into liver and carcass lipids and cholesterol in biotin-deficient rats. J. Nutr., *64:* 217.
23. Kukral, J. C., A. P. Adams and F. W. Preston 1965 Protein-producing capacity of the human exocrine pancreas. Ann. Surg., *162:* 63.

Effect of Diet on Protein Synthesis and Nucleic Acid Levels in Rat Liver [1]

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ABSTRACT The effects of variation in dietary protein content on the rates of liver protein and plasma albumin synthesis have been reinvestigated. Adult rats were preconditioned to normal (27%), high (64%), low (8%) and protein-free diets for 5 to 7 weeks. Following a 4-hour fast they were injected with DL-lysine-1-^{14}C intravenously at different time intervals up to 2 hours before killing. Determinations were made of DNA, RNA, protein, and free lysine concentrations in the liver, and of the specific activity of lysine in the protein-bound and TCA-soluble fractions. The rate of liver protein synthesis remained relatively unchanged in low and high protein diet groups, as compared with the normal protein diet group, and was either unchanged or slightly increased in the animals on the protein-free diet. On the other hand, the rate of plasma albumin synthesis was significantly reduced in protein deprivation, although it did not show any appreciable differences among other groups. DNA, content was found to be higher in the liver of rats fed the protein-free diet, as compared with those on normal diet, whereas RNA concentration was reduced considerably, the differences among other groups being small. Protein content of the liver changed proportionate to the protein content of the diet. Free lysine concentration in the liver was found to be relatively unaffected by dietary treatment.

Although the effect of the protein content of the diet on protein levels and on protein turnover in the liver of rats has been investigated by many workers, the results are conflicting. Most investigators have utilized labeled amino acids in their work, and unknown degrees of reincorporation of the label have been the major source of difficulty in interpretation, as for instance in the studies of Solomon and Tarver (1). Attempts have been made to circumvent this difficulty by investigating the behavior of an amino acid labeled in a group known to be vulnerable to metabolic attack, namely, the guanidino group of arginine (2, 3). The results showed that the turnover in this group was not influenced until the level of dietary protein was reduced to a level barely compatible with life. A refinement of this method was adopted by Stephen and Waterlow (4) who used arginine labeled in both the C-6 and C-1 groups. Their protein-depleted animals exhibited a decrease in protein catabolic rate but, as pointed out by Lane (5), it is possible that their results should be ascribed to alterations attributable to the surgical manipulations involved. An increase in the rates of protein synthesis in livers of protein-deficient rats has also been reported (6, 7).

There are also conflicting data with regard to the effect of diet on the turnover of the mixed plasma proteins as well as that of plasma albumin. Yuile et al. (8) observed that turnover rates of both plasma albumin and globulins increased when the level of dietary protein was raised whereas Jeffay and Winzler (9), using endogenously labeled plasma proteins, reported that the turnover rate of albumin, but not of globulins was dependent on the diet. Kirsch et al. (10) recently made more elaborate studies, measuring catabolism by the excretion of ^{131}I from the breakdown of intravenously injected labeled albumin and synthesis from the incorporation of ^{14}C into the guanidino carbon of arginine in albumin. A reduction in both anabolic and catabolic rates was found although the change in anabolic rate was detected first.

In view of the above mentioned conflicting results and others cited in the literature, we decided to reinvestigate this

Received for publication June 12, 1969.
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problem, giving particular attention to the dietary state of our animals and to the specific activity of the labeled amino acid (lysine) during the period in which incorporation was studied. We also investigated the protein and RNA levels in the livers of our experimental animals. Because in the adult organism the DNA of the nucleus appears to be the least variable of all tissue constituents, DNA concentrations were also determined as a standard of reference for all other cell components.

MATERIALS AND METHODS

Animals and treatment. Female rats of the Sprague-Dawley strain weighing about 200 g were housed in individual cages and fed diets [3] for 5 to 7 weeks. Their daily food consumption on high and low protein diets was not significantly different from those on normal protein diet (15 to 18 g), whereas rats on the protein-free diet consumed more food than those on the normal diet initially but less later on (12 to 14 g). Rats on normal, low and high protein diets gained at the average rates of 12.5, 6.5, and 12.1 g per week, respectively, while those on protein-free diet lost 13.5 g per week.

Determination of blood in liver. A portion of the liver homogenate was diluted and used for blood determination by a modified benzidine method (11) in order to make allowance for its radioactivity. A sample of heparinized blood obtained at the time of killing served, after dilution, as the standard. Synthesis of liver protein was measured as follows: Rats fasted for 4 hours in the morning were injected through the tail vein with 20 μCi of DL-lysine-1-^{14}C [4] in 0.5 ml saline. After each of the six time intervals of 5 to 120 minutes, four to six rats were decapitated, their livers were removed and homogenized separately in ice-cold water. An aliquot was removed and the rest precipitated with 0.5 volume of 20% trichloroacetic acid (TCA).

Treatment of the homogenate. The liver homogenate precipitated with TCA was centrifuged, and the centrifugate rehomogenized with 10% TCA. The combined supernatant and wash was used for the determination of free lysine, after removal of TCA by repeated extractions with ether and concentration to a small volume. RNA, DNA and protein were determined on the precipitate by the procedure of Hutchinson and Munro (12), but using the method of Ceriotti (13) for DNA and biuret (14) or Lowry et al. (15) for protein.

Determination of specific activity of lysine in the TCA-soluble fraction and in liver protein. Labeled liver protein precipitated with TCA was prepared for determination of specific activity of lysine as follows: Nucleic acids were removed from the precipitate by extraction with 10% TCA at 90° for 15 minutes (Schneider method (16)). Lipid and TCA were removed by extraction, twice with 95% ethanol, twice with ethanol–ether (3:1) at 40° for 10 minutes and finally twice with ethyl ether. One hundred milligrams of the dry residue was hydrolyzed in a sealed tube with 3 ml 6 N HCl for 22 hours at 105 to 110°. The acid was removed by repeated evaporation and dilution with water in a flash evaporator. The pH of the final concentrate was made to 6.0 with a solution of sodium hydroxide.

For the determination of lysine, either one of two methods was used, depending upon the requirements. Electrophoresis in a vertical system at 220 volts for 22 hours, using 0.05 M sodium carbonate (pH 11.3) in the buffer chambers, along with suitable standards, or a manometric method using lysine decarboxylase [5] as described by Gale (17).

The measurement of radioactivity in the form of lysine in both TCA-soluble fractions and protein hydrolysates was also done using the decarboxylase, by a modification of the method of Snyder and Godfrey (18). An aliquot of the hydrolysate or diluted TCA-soluble extract was placed in the reaction compartment of a Warburg flask with two side arms, and the volume was made to 2.0 ml by the addition of 0.5 M phosphate buffer at pH 6.0. To one side arm was added 5 mg lysine decarboxylase in 0.5 ml phosphate buffer,

[3] Composition of diets in percent: *Normal* (N), casein (vitamin free) 27, starch 59, vegetable oil 10, salt mixture (USP XIV) 4, plus vitamin fortification mixture; *Low* (L), casein 8, starch 78, vegetable oil 10, salt mixture 4, and vitamins; *High* (H), casein 64, sucrose 22, vegetable oil 8, brewer's yeast (USP) 2, salt mixture (USP no. 2) 4, plus vitamins; *Free* (F), cornstarch 70, cellulose 15, vegetable oil 10, salt mixture 4, and vitamins. All diets were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Specific activity 15.5 mCi/mmole. Nuclear Chicago Corporation, Des Plaines, Illinois.

[5] B grade, CalBiochem, Los Angeles, California.

and to the other 0.25 ml 8 N H_2SO_4. A tube, 3 cm long, made of 7-mm glass tubing and containing 0.3 ml hydroxide of hyamine [6] was placed in the central well. After all outlets to the flask were closed, the enzyme was tipped into the reaction compartment and the mixture was incubated in a Dubnoff's shaker at 37° for 1 hour. The acid was then tipped in and incubation continued for 2 hours, after which the hyamine-containing tubes were placed in counting vials with 10 ml of Bray's solution. The contents were mixed and counted in a Packard TriCarb liquid scintillation counter. Corrections for quenching were made by using an automatic external standard.

The synthesis rate of plasma albumin was measured 60 and 120 minutes after injection of the labeled lysine. Albumin labeled with ^{125}I [7] of known activity was injected simultaneously in order to measure blood and plasma volumes by isotope dilution. Albumin concentration in plasma was determined by the method of Debro et al. (19).

At the conclusion of the experimental interval, blood was collected by heart puncture and the albumin was prepared from the plasma by the method used by Awwad et al. (20). Purity of the albumin was checked by acrylamide gel electrophoresis.[8] The specific activity of lysine in albumin was determined in the manner described for liver protein. Aliquots of plasma samples from rats killed at 60 or 120 minutes were counted for ^{125}I activity in order to get an estimate of the fraction of labeled albumin lost by passage into extravascular space or catabolism.

RESULTS

Liver DNA, RNA, protein and free lysine concentrations. DNA is expressed as mg/10 g wet liver, mg/200 g initial body weight and mg/200 g final body weight. RNA and protein concentrations are in addition, expressed as mg/10 mg DNA and g/10 mg DNA, respectively. Student's *t* test was used to assess the significance of the difference between the mean values of two groups of data. Differences were accepted as significant only when $P < 0.01$.

No significant differences were found in the DNA content of the liver from low or high protein-fed rats, compared with the controls, when expressed either as mg/200 g initial body weight or mg/200 g final body weight (fig. 1). On the other hand the protein-free diet group showed a lower DNA concentration by the first mode of expression, and a higher by the second. DNA as mg/10 g wet liver was higher in both the low and protein-free diet groups, in comparison with results from rats on normal protein diet, and showed statistically insignificant differences in the case of the high-protein diet group. Several reports have indicated that in the adult rat, DNA content of the liver is relatively unaffected by dietary manipulations (21–23). Other workers (24–26), however, have reported higher DNA content of the livers from rats fed a protein-free diet for varying periods of time. Our results are in agreement with the findings of the latter group. Nevertheless, DNA has been used as the standard of reference, with the knowledge that results may be somewhat underestimated for the protein-free diet group when this mode of expression is used.

Considerably lower concentrations of RNA were found in livers of rats fed the protein-free diet, when normal protein diet was taken as standard of comparison. The differences between the high and normal, and normal and low protein diet groups were not so marked and did not consistently show the same pattern of change with all four means of expression, although a trend towards more RNA with increase in dietary protein level was evident (fig. 2). Protein concentration in the liver was found to increase with increase in the protein content of the diet (fig. 3).

Figure 4 shows the free lysine concentration in the livers of rats on the four diets, expressed as mg/10 g wet liver, mg/200 g final body weight, and mg/10 mg DNA. No statistically significant differences were found for rats fed the high, low or protein-free diet as compared to the normals, when lysine content of the liver was expressed as mg/10 g wet liver. The

[6] This and other materials for counting were obtained from Packard Instrument Company, Inc., La Grange, Illinois.

[7] Free of carrier and reducing agent. Nuclear Chicago Corporation, Des Plaines, Illinois.

[8] Polyacrylamide gel materials: E–C Apparatus Corp., Philadelphia, Pennsylvania.

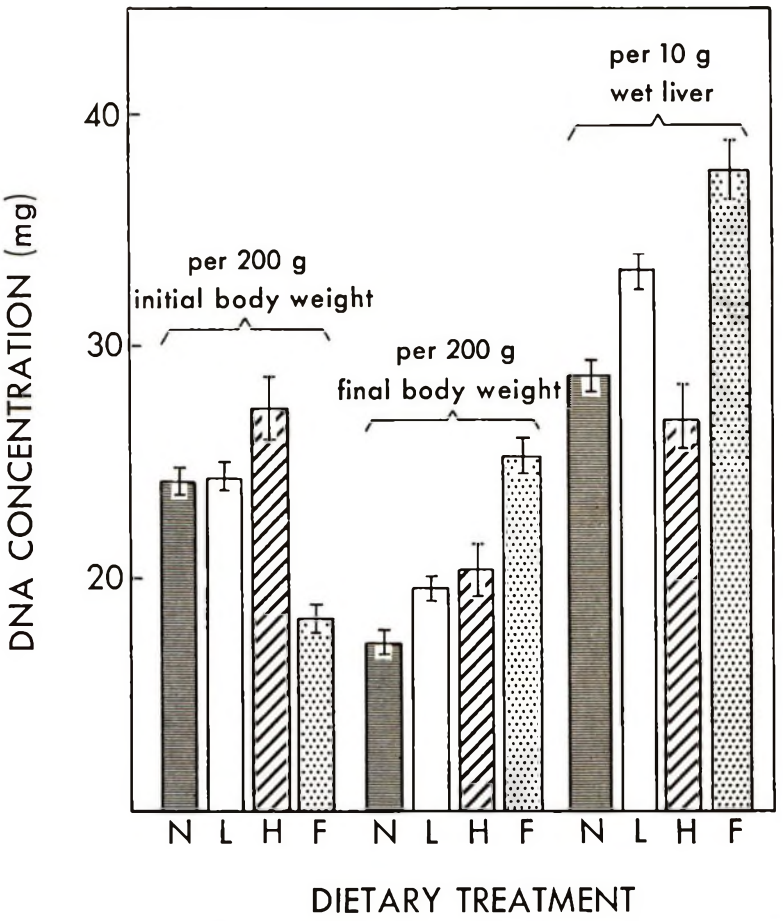

Fig. 1 Effect of dietary protein variation on DNA content of the liver. Rats were preconditioned to normal (N), low (L), high (H) or no-protein (F) diets for 5 to 7 weeks before the DNA contents of the livers were determined as described in Methods. Values represent the mean of 23 to 31 rats ± SEM.

differences found with the other two modes of expression are small, and we conclude that free lysine concentration in the liver remains constant despite wide variations in protein intake.

Our average values for lysine content of the liver (mg/10 g) agree reasonably well with those reported by Schurr et al. (27) and Gaetani et al. (6), but are considerably lower than the reported values of Solomon and co-workers (28).

The average lysine content of liver protein from rats maintained on the four diets is shown in table 1. No significant differences were found for any group. Dietary protein variation does not alter the lysine content of liver protein. The average values for lysine content of liver protein found by us are in agreement with those reported by Solomon et al. (28).

Specific activity of lysine in the TCA-soluble fraction, liver protein and plasma albumin. Specific activities have been expressed as percentage of injected dose per milligram lysine, corrected for variation in body weight by adjustment to a 200 g final body weight.

The changes in specific activity of free lysine in liver with time are shown in figure 5. In all four dietary groups, the peak specific activity was attained 5 minutes after injection and the curves leveled off after 40 minutes. The maximum specific activity of lysine in liver protein (fig. 6)

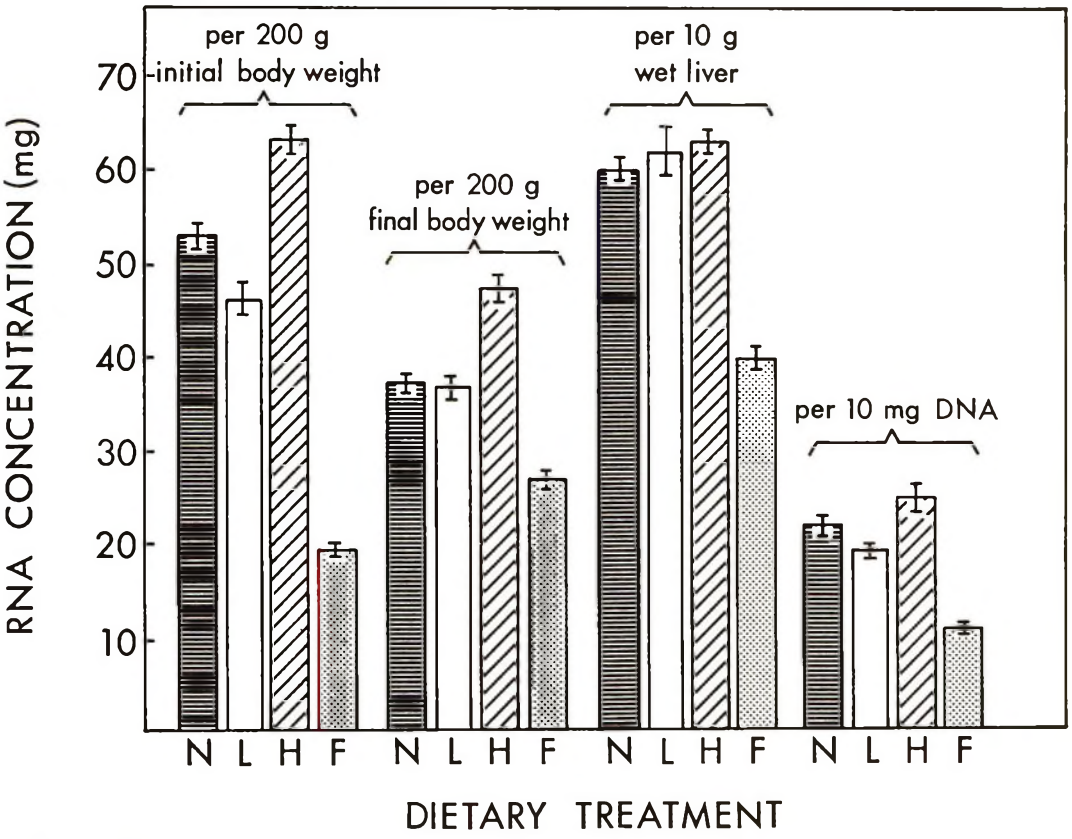

Fig. 2 Effect of dietary protein variation on RNA content of the liver. These data were obtained from the same rats as in figure 1.

was reached after 40 to 60 minutes, after which the curves reached a plateau. The specific activity of lysine in liver protein and plasma albumin (fig. 7) varied inversely with the protein content of the diet, the highest specific activity being for liver proteins from rats fed a protein-free diet and the lowest for those fed high protein diet. This pattern of labeling has been observed by several previous workers (1, 29).

Plasma volumes were not found to be significantly different in rats from the four dietary groups (table 2). Plasma albumin concentration (mg/ml) in the normal, low, and high protein diet groups was approximately the same and, therefore, the total circulating albumin in these groups was also substantially the same. In animals on a protein-free diet, albumin concentration in the plasma and hence the total circulating albumin was considerably reduced.

Rate of synthesis of liver protein and plasma albumin. The rate of synthesis of liver protein was calculated using a formula similar to the one used by Richmond et al. (30):

$$S_L = \tfrac{1}{2} \cdot \frac{V}{C} \int_0^2 S_A \, dt - \int_0^2 S_{Pr} \, dt ,$$

where:

S_L = the specific activity of lysine in the liver protein at 2 hours;

S_A = the specific activity of precursor-free lysine in the liver over the 2-hour interval;

S_{Pr} = the specific activity of lysine in liver protein over the 2-hour period;

V = the rate of protein synthesis in liver expressed in grams/2 hours per 200 g body weight;

= concentration of protein in liver expressed in grams/200 g final body weight.

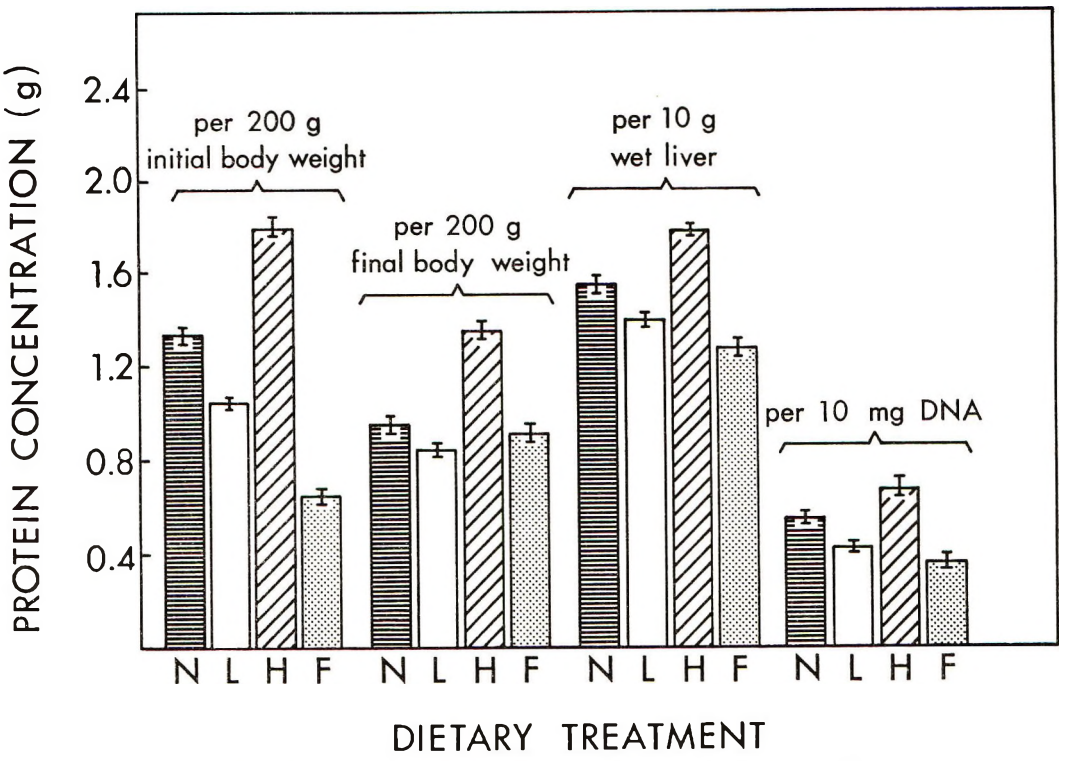

Fig. 3 Effect of dietary protein variation on protein content of the liver. These data were obtained from the same rats as in figure 1.

The values of the integrals $\int_0^2 S_A\,dt$ and $\int_0^2 S_{Pr}\,dt$ were obtained graphically from the respective specific activity curves. The rates of protein synthesis found are given in table 3.

For calculation of rate of plasma albumin synthesis, the formula suggested by Awwad et al. (20) was used: If the fraction of injected lysine incorporated into plasma albumin during the 2-hour period is represented by F, and

$$F = a\,[\,s_2 + k\int_0^2 s_t\,dt\,]\,p,$$ where:

s_2 = specific activity of lysine in albumin at 2 hours;

a = milligrams of lysine per gram of albumin (the reported value of 128.3 (31) was used);

k = the fraction of labeled albumin lost from the blood circulation during 2 hours, determined by the use of ^{125}I-labeled albumin;

s_t = the specific activity of lysine in circulating albumin during the 2-hour interval (The value for $\int_0^2 s_t\,dt$ was obtained by graphic integration of the albumin lysine specific activity curve, $k\int_0^2 s_t\,dt$ is a term added to the measured specific activity of serum albumin at 2 hours to correct for the loss of labeled albumin from the circulation during the 2-hour period);

p = total circulating albumin;

Then: the rate of albumin synthesis in grams per hour = $F/2aS$, in which S = mean specific activity of free lysine in the liver, calculated from the curve by graphic integration, over the 2-hour period.

DISCUSSION

The literature on the effects of variation in dietary protein content on the turnover rates of liver protein and plasma albumin is rather discrepant and far from being conclusive. In most such studies, in which measurements of incorporation of amino acids were made to assess the turnover of

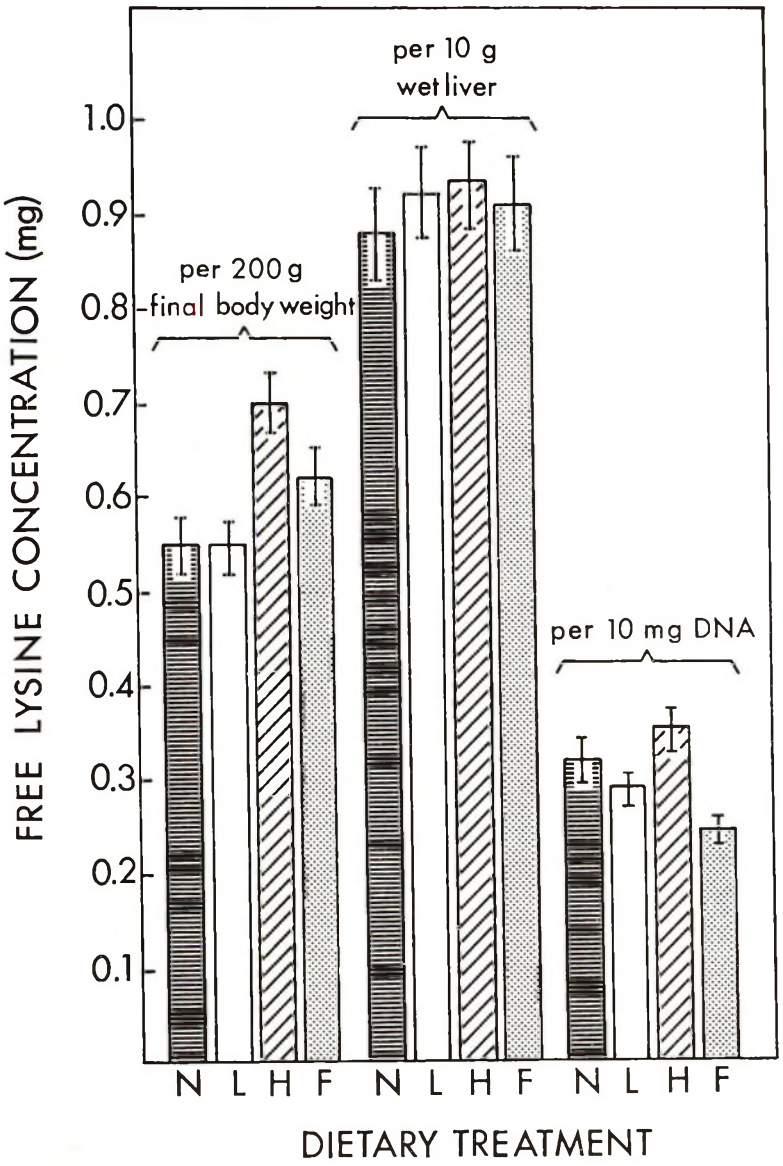

Fig. 4 Effect of dietary protein variation on free lysine concentration of the liver. These data were obtained from the same rats as in figure 1.

liver protein, information is lacking about the specific activity of the precursor amino acid, liver free amino acid and the size of the protein pool. Isolated measurements of protein specific activity are insufficient to yield quantitative information about rates of protein synthesis, and the need for knowledge of the specific activity of the precursor amino acid pool and how it changes with time is particularly evident in the case of turnover studies in dietary protein deprivation, where reutilization of the label assumes a dominant role. This makes the interpretation of results difficult. The aim of the present study was to re-investigate the problem of effect of diet on the rates of liver protein and albumin synthesis, circumventing the criticisms re-

TABLE 1

Effect of dietary protein variation on lysine content of liver protein [1]

Diet	Lysine content of liver protein
	mg/100 mg
Normal protein	7.14 ± 0.249 [2]
Low protein	7.19 ± 0.218
High protein	6.87 ± 0.231
No protein	6.91 ± 0.259

[1] Data were from the same animals used in figure 1.
[2] Mean of 23 to 31 rats ± SEM.

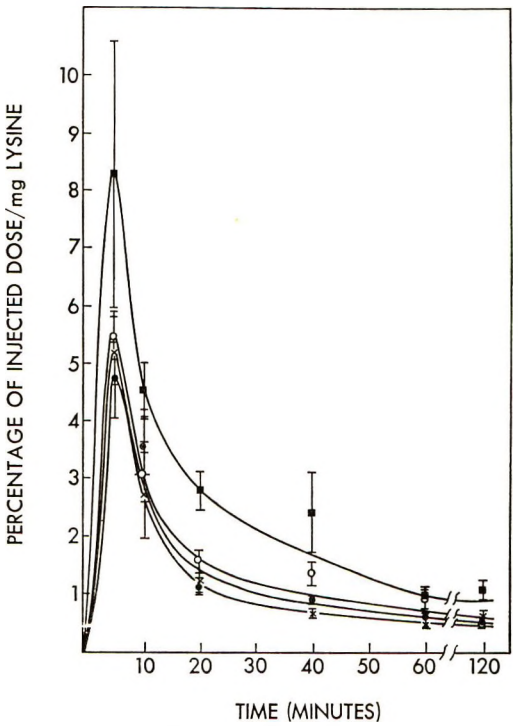

Fig. 5 Specific activity of free lysine in liver at different time intervals. These data were obtained from the same animals as in figure 1, and specific activity of free lysine was determined as described in Methods. Specific activity values have been adjusted to a 200 g body weight. Points represent mean of 4 to 6 animals ± SEM. ——○——, Normal protein diet. ——●——, Low protein diet. ——×——, High protein diet. ——■——, Protein-free diet.

ferred to above. By studying incorporation over a short time-interval relative to the half-life of liver protein, it has been possible not only to eliminate the complication of reutilization of the label, but also to observe changes in protein synthetic rates separated from catabolic rates.

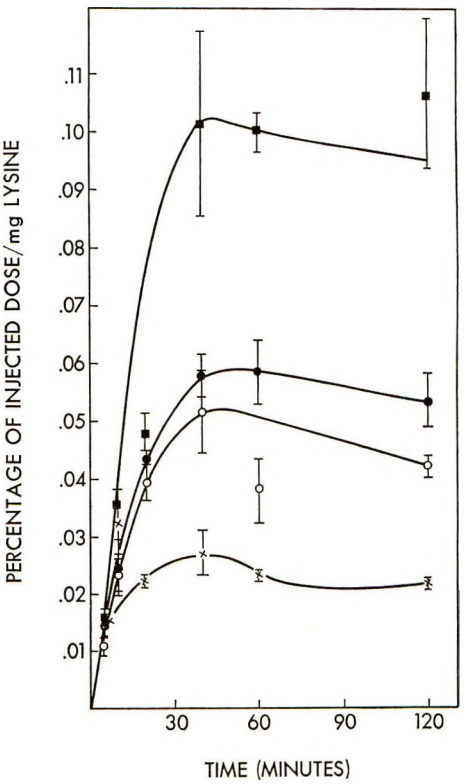

Fig. 6 Specific activity of lysine in liver protein at different time intervals. These data were obtained from the same animals as in figure 1. Specific activity of lysine in liver protein was determined as described under Methods, and its value adjusted to a 200 g body weight. Points represent mean of 4 to 6 animals ± SEM. ——○——, Normal protein diet. ——●——, Low protein diet. ——×——, High protein diet. ——■——, Protein-free diet.

In our studies, RNA was estimated with a view to observing how changes in its concentration with dietary variation correlate with changes in protein synthetic rates, and DNA was estimated to serve as standard of reference for other cell components. DNA concentration in the liver of rats fed the protein-free diet showed an opposite effect, using mg/200 g initial body weight and mg/200 g final body weight as means of expression, being lower than normal by the former and higher than normal by the latter method. This is explainable by the fact that rats on normal, low and high protein diets were constantly increasing in their body weights, whereas those on protein-free diet were losing

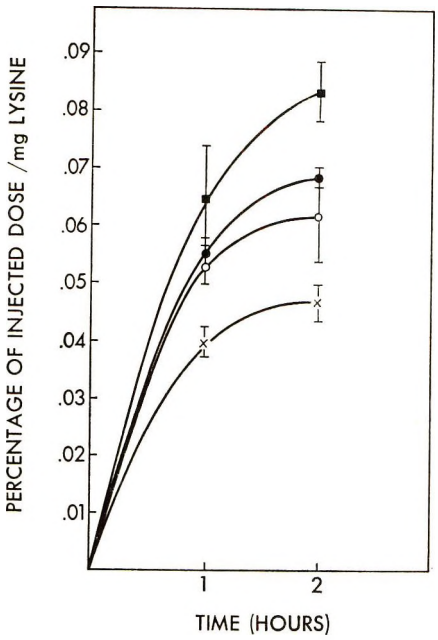

Fig. 7 Specific activity of lysine in albumin at two time-intervals. These data were obtained from rats treated in the same way as in figure 1. Specific activity of lysine in albumin was determined as described in the text, and adjusted to a 200 g body weight. Points represent mean of 4 to 5 animals ± SEM. ——○——, Normal protein diet. ——●——, Low protein diet. ——×——, High protein diet. ——■——, Protein-free diet.

weight. Since DNA in the liver increases with increase in body weight (21), the calculation of DNA concentration per 200 g initial body weight will yield a lower value for the protein-free group, in which there was a reduction in body weight, compared with rats on the other three diets, which gained in body weight. When this increase is taken into account (mg/200 g final body weight) and when expressed as mg/10 g wet liver, the DNA content in the liver showed significantly higher values for the protein-free group, compared with the controls fed normal protein diet. Seifter et al. (25), who made similar observations, attributed the higher DNA content to increased cell density.

The decreased liver RNA content of rats on protein-free diet, observed by us, is attributable to decreased breakdown (32). The absence of clear-cut and consistent differences among other groups may perhaps be an indication of the fact that rates of protein synthesis were relatively unaffected in these groups. Moreover, since even after a short fast like the one used in our experiments, there is considerable breakdown of RNA, it is possible that differences between RNA content in the liver from normal, low and high protein groups were somewhat masked.

The dietary manipulations apparently did not cause any significant changes in the free lysine content of the liver. The reports in the literature on the effect of diet on the free amino acid content of tissues are discrepant. Thompson et al. (33) reported no change in the free lysine content of liver and only a 20% reduction in the muscle in the adult rats fed a protein-free diet for 3 weeks. Roberts and Simonsen (34), studying the effects of starvation for 9 days, arrived at the same conclusion. Similar results were also reported by Waterlow and Stephen (35) and Schimke (36). On the other hand, Allison et al. (23) observed a considerable fall in the free lysine concentration of both liver and

TABLE 2

Effect of dietary protein variation on plasma volume, plasma albumin concentration and total circulating albumin

Diet	Plasma volume	Plasma albumin	Total circulating albumin
	ml/200 g body wt [1]	*mg/ml* [2]	*mg/200 g body wt* [3]
Normal protein	6.26	31.1 ± 1.9	200
Low protein	6.44	30.9 ± 1.3	199
High protein	6.70	30.0 ± 1.2	193
No protein	6.42	19.0 ± 1.7	122

[1] Average of 8 to 10 rats.
[2] Average of 8 to 10 rats ± SEM.
[3] Calculated on the basis of a value of 6.45 ml for plasma volume per 200 g body weight found as an average of 35 rats.

TABLE 3

Rate of synthesis of liver protein and plasma albumin

Diet	Liver protein	Plasma albumin
	mg/hr per 200 g body weight	
Normal protein (N)	17.1	7.52
Low protein (L)	23.2	9.55
High protein (H)	15.6	6.79
Protein free (F)	26.2	3.73

muscle, in rats fed a protein-free diet for 3 days.

The constancy of the intracellular pool of lysine emphasizes the role of endogenous protein metabolism in the body economy, pointed out by Nasset (37). This concept has recently been supported by the observation of Gan and Jeffay (38) that protein catabolism in normal and starved rats contributed approximately 50% and 90% of the liver amino acids, respectively. They also noted that the liver, muscle and plasma lysine concentrations remained unchanged during a starvation period of 7 days.

It is difficult to decide with certainty whether the values obtained for the rate of liver protein synthesis (table 3) are indicative of a real difference among the dietary groups. The high variability among animals, as shown by rather large standard deviations, and the possibility that rate of protein synthesis varies from time to time and individual to individual, are two considerations pointing to the difficulties in arriving at clear-cut conclusions. Another is the mode of expression of protein concentration for use in calculations (g/200 g final body weight), which had to be adopted since all specific activities were adjusted to a 200 g final body weight. The final body weight is at least partly conditioned by changes in the lipid content of the body during the feeding periods and is therefore less uniform. We do not attach any significance to the higher rate of protein synthesis for the low protein diet group as compared to normal, since the value of the integral $\int_0^2 S_A\, dt$ for this group was found to be lower than that for the normal, when it should be higher or at least equal to it. The anomaly may be due to experimental error or variability among the individual animals. If this value is assumed to be equal to that for the normal group, the difference in the rates of synthesis between the two groups is considerably reduced. The same reasoning applies to the higher rate for plasma albumin synthesis for this group (see below). Within the limitations imposed by the considerations alluded to above, we conclude that the rate of protein synthesis in the liver is not substantially different for the normal, low and high protein-fed animals, whereas in rats on protein-free diet, it is either unchanged or somewhat increased.

It is of interest in this connection to consider the results of Schimke (39) on the effect of protein content of the diet on the activity of certain liver enzymes. He noted a reduction in the activity of most of the urea cycle enzymes, in animals on a protein-free diet. On the other hand, Mariani et al. (40) and Kean (41) found higher activities of some amino acid-activating enzymes in the livers of rats fed a protein-free diet. As pointed out by Waterlow and Stephen (35) these changes, taken together, might produce the condition whereby an amino acid molecule in the liver would have a lesser chance of being catabolized and a greater opportunity of being reincorporated into protein. This provides an explanation for the paradoxical situation that in protein depletion the rate of incorporation of radioactivity in the liver protein was normal or greater than normal, but the loss of label was considerably slowed down (1, 29). Because of the latter finding, it has been suggested in the past that the turnover of liver proteins is decreased in such dietary conditions. It is of interest, however, to note that in the few studies in which the problem of reutilization has been circumvented either by the use of arginine-6-^{14}C which is not reutilized (3), or by indirect determination of precursor specific activity using continuous feeding of ^{14}C-bicarbonate (2), or by the continuous infusion method keeping the specific activity of the amino acid constant (42), no significant effects of dietary treatment on turnover rate of liver protein have been observed. Our studies, using direct determination of the precursor amino acid specific activity, support these findings and lead us to state that protein synthesis in

the liver operates at almost normal rate, despite wide variations in the protein content of the diet. A somewhat similar suggestion has been made by Waterlow and Stephen (35). The effect on the catabolic rate, and an increased and more efficient reutilization of the label may be the chief regulatory mechanisms in conditions of protein deprivation. That protein synthetic and degradative rates can be affected independently of each other has been noted in the case of some enzymes such as tryptophan pyrrolase (43), and both may be important in the control of the level of a specific enzyme or protein.

As regards the effect of diet on albumin metabolism, there is reasonably good evidence in the literature that both absolute and fractional catabolic rates are reduced in protein depletion (44–46) and this reduction appears to be related to decrease in albumin pool. Whether or not absolute rate of albumin synthesis is likewise changed in protein depletion has remained an open question, because of the lack of studies in which determination of the precursor amino acid specific activity was made for calculation of synthetic rate. Our studies using this method show a definite reduction in the rate of albumin synthesis in rats given the protein-free diet. The explanation for high rate of synthesis in the case of the low protein diet group has already been given. Kirsch et al. (10) also have recently reported an even greater decrease in albumin synthesis in rats on a protein-free diet, using relative rates of incorporation of ^{14}C into urea and into the guanidino carbon of arginine.

We believe that the response of the animals to a diet which differs from normal in composition varies with the length of time the diet is fed. The labile protein reserves of the liver are lost or gained rapidly following alteration in the nutritional state of the animal (32). After a period of time, however, a fairly steady level of protein in the liver is achieved, and changes in its concentration are greatly slowed down. Therefore, during short-term treatments when the body tries to adjust to the new nutritional state, wide variations in the amino acid pools, and in the rates of protein synthesis probably take place. Prolonged treatment, however, brings into play many adjustment mechanisms which help keep the intracellular amino acid level constant and the rate of protein synthesis close to normal. This can explain the differences in liver protein concentrations in rats fed diets of different protein composition, although the effect on the rate of protein synthesis in the liver is small.

ACKNOWLEDGMENT

The authors are pleased to thank Miss Leila Kotite for her excellent technical assistance.

LITERATURE CITED

1. Solomon, G., and H. Tarver 1952 The effect of diet on the rate of loss of labeled amino acid from tissue protein. J. Biol. Chem., *195:* 447.
2. Swick, R. W. 1958 The use of isotopes in dynamic equilibria studies of protein synthesis. Proc. Second U. N. Int. Conf. Peaceful Uses At. Energy, 25: 115.
3. Schimke, R. T. 1964 The importance of both synthesis and degradation in the control of arginine levels in rat liver. J. Biol. Chem., 239: 3808.
4. Stephen, J. M. L., and J. C. Waterlow 1966 Use of carbon-14-labelled arginine to measure the catabolic rate of serum and liver proteins and the extent of amino-acid recycling. Nature, *211:* 978.
5. Lane, R. S. 1967 Use of carbon-14-labelled arginine to measure catabolic rates of proteins and the recycling of amino-acids. Nature, *214:* 1331.
6. Gaetani, S., A. Mariani, M. A. Spadoni and G. Tomassi 1961 Distribuzione della C^{14}-lizina nel plasma, fegato, cuore, e muscolo gastrocnemio di ratti in deplezione proteica. Boll. Soc. Ital. Biol. Sper., 37: 1685.
7. Schreier, K., and C. Kazassis 1960 Influence of different types of malnutrition on the ^{14}C-lysine metabolism in young rats. Nature, *187:* 1117.
8. Yuile, H., F. V. Lucas, J. P. Olson and A. B. Shapiro 1959 Plasma-protein and tissue exchange—influence of dietary protein and protein depletion. J. Exp. Med., *109:* 173.
9. Jeffay, H., and R. J. Winzler 1958 The metabolism of serum proteins. II. The effect of dietary protein on the turnover of rat serum protein. J. Biol. Chem., 231: 111.
10. Kirsch, R., L. Frith, E. Black and R. Hoffenberg 1968 Regulation of albumin synthesis and catabolism by alteration of dietary protein. Nature, *217:* 578.
11. Crosby, W. H., and F. W. Furth 1956 A modification of the benzidine method for measurement of hemoglobin in plasma and urine. Blood, *11:* 380.
12. Hutchinson, W. C., and H. N. Munro 1961 The determination of nucleic acids in biological materials. Analyst, 86: 768.

13. Ceriotti, G. 1952 A microchemical determination of desoxyribonucleic acid. J. Biol. Chem., *198:* 297.
14. Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by the biuret reaction. J. Biol. Chem., *177:* 751.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
16. Schneider, W. E. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.
17. Gale, E. F. 1945 Studies on amino-acid decarboxylases. 5. The use of specific decarboxylase preparations in the estimation of amino-acids and in protein analysis. Biochem. J., *39:* 46.
18. Snyder, F., and P. Godfrey 1961 Collecting $C^{14}O_2$ in a Warburg flask for subsequent scintillation counting. J. Lipid Res., *2:* 195.
19. Debro, J. R., H. Tarver and A. Korner 1957 The determination of serum albumin and globulin by a new method. J. Lab. Clin. Med., *50:* 728.
20. Awwad, H. K., S. J. Adelstein, E. J. Potchen and J. B. Dealy, Jr. 1967 The interconversion and reutilization of injected ^{75}Se-selenomethionine in the rat. J. Biol. Chem., *242:* 492.
21. Campbell, R. M., and H. W. Kosterlitz 1950 The effects of growth and sex on the composition of the liver cells of the rat. J. Endocrinol., *6:* 308.
22. Thomson, R. Y., F. C. Heagy, W. C. Hutchinson and J. N. Davidson 1953 Deoxyribonucleic acid (DNA) content of the rat-cell nucleus and its use in expressing the results of tissue analysis, with particular reference to the composition of liver tissue. Biochem. J., *53:* 460.
23. Allison, J. B., R. W. Wannemacher, Jr. and W. L. Banks, Jr. 1963 Influence of dietary proteins on protein biosynthesis in various tissues. Federation Proc., *22:* 1126.
24. Muntwyler, E., S. Seifter and D. M. Harkness 1950 Some effects of restriction of dietary protein on the intracellular components of liver. J. Biol. Chem., *184:* 181.
25. Seifter, S., E. Muntwyler and D. M. Harkness 1951 Some effects of continued protein deprivations with and without methionine supplementation, on intracellular liver components. Proc. Soc. Exp. Biol. Med., *75:* 46.
26. Ely, J. O., and M. H. Ross 1951 Desoxyribonucleic acid content of rat liver nuclei influenced by diet. Science, *114:* 70.
27. Schurr, P. E., H. T. Thompson, L. M. Henderson, J. N. Williams, Jr. and C. A. Elvehjem 1959 The determination of free amino acids in rat tissues. J. Biol. Chem., *182:* 39.
28. Solomon, J. D., C. A. Johnson, A. L. Sheffner and O. Bergeim 1951 The determination of free and total amino acids in rat tissues. J. Biol. Chem., *189:* 629.
29. Muramatsu, K., T. Sato and K. Ashida 1963 Dietary protein level and the turnover of tissue protein in rats. J. Nutr., *81:* 427.
30. Richmond, J. E., W. C. Shoemaker and D. H. Elwyn 1963 Rates of biosynthesis of plasma and liver proteins. Amer. J. Physiol., *205:* 848.
31. Phelps, R. A., and F. W. Putnam 1960 Chemical composition and molecular parameters of purified plasma proteins. In: The Plasma Proteins, vol. 1, ed., F. W. Putnam. Academic Press, New York, p. 143.
32. Munro, H. N. 1964 General aspects of the regulation of protein metabolism by diet and by hormones. In: Mammalian Protein Metabolism, vol. 1, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 381.
33. Thompson, H. T., P. E. Schurr, L. M. Henderson and C. A. Elvehjem 1950 The influence of fasting and nitrogen deprivation on the concentration of free amino acids in rat tissues. J. Biol. Chem., *182:* 47.
34. Roberts, E., and D. G. Simonsen 1962 Free amino acids in animal tissues. In: Amino Acid Pools, ed., J. T. Holden. Elsevier Publishing Co., Amsterdam, p. 334.
35. Waterlow, J. C., and J. M. L. Stephen 1966 Adaptation of the rat to a low-protein diet. The effect of a reduced protein intake on the pattern of incorporation of L-lysine-^{14}C. Brit. J. Nutr., *20:* 461.
36. Schimke, R. T. 1963 Studies on factors affecting the levels of urea cycle enzymes in rat liver. J. Biol. Chem., *238:* 1012.
37. Nasset, E. S. 1956 Essential amino acids and nitrogen balance. In: Some Aspects of Amino Acid Supplementation, ed., W. H. Cole. Rutgers University Press, New Brunswick, New Jersey, p. 3.
38. Gan, J. C., and H. Jeffay 1967 Origins and metabolism of the intracellular amino acid pools in rat liver and muscle. Biochim. Biophys. Acta, *148:* 448.
39. Schimke, R. T. 1962 Differential effects of fasting and protein-free diets on levels of urea cycle enzymes in rat liver. J. Biol. Chem. *237:* 1921.
40. Mariani, A., M. A. Spadoni and G. Tomassi 1963 Effect of protein depletion on amino acid-activating enzymes of rat liver. Nature, *199:* 378.
41. Kean, E. A. 1966 Effect of methionine-deficient diet on amino acid incorporation in rat liver cell fractions. J. Nutr., *90:* 91.
42. Stephen, J. M. L., and J. C. Waterlow 1965 Protein turnover in the rat measured by constant infusion of lysine-^{14}C. J. Physiol. (London), *178:* 40P.

43. Schimke, R. T., E. M. Sweeney and C. M. Berlin 1965 The roles of synthesis and degradation in the control of rat liver tryptophan pyrrolase. J. Biol. Chem., *240:* 322.
44. Picou, D., and J. C. Waterlow 1962 The effect of malnutrition on the metabolism of plasma albumin. Clin. Sci., *22:* 459.
45. Cohen, S., and J. D. L. Hansen 1962 Metabolism of albumin and γ-globulin in kwashiorkor. Clin. Sci., *23:* 351.
46. Hoffenberg, R., E. Black and J. F. Brock 1966 Albumin and globulin tracer studies in protein depletion states. J. Clin. Invest., *45:* 143.

Rat Liver Glycogen-lowering Activity of Fed Creatine — A Retraction [1,2]

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ABSTRACT Earlier studies led us to report that feeding rats a semipurified diet containing 1% creatine for 20 hours brought about a marked decrease in liver glycogen (from 4% to 1% wet weight). The Practical Grade creatine (Eastman No. P951, Lot 12) was found to contain a readily separable impurity, 1,1-dimethylbiguanide which was responsible for this action and highly active at a level of 0.02% of the diet. Authentic samples of the biguanide as well as a number of other guanidine derivatives were found to possess this physiological action when fed at a level of 0.01% of the ration. The purified creatine (three recrystallizations) and other creatine samples had no such effect when fed under the same conditions. Dimethylbiguanide injected intraperitoneally caused a precipitous drop in liver glycogen in the following 4 hours. From these data and the reports of other workers it is concluded that the results are due to drug inhibition of gluconeogenesis.

In a previous communication (1) it was reported that liver glycogen levels were markedly decreased in rats prefed for 20 hours on a semipurified diet containing 1% added creatine. Sometime later this depression of liver glycogen could not be obtained under the same conditions except that the creatine employed in the later experiments was from a different lot than that used earlier.[3]

It is now clear than an impurity—easily separated from the original "active" creatine—accounted for the low glycogen levels. The "purified" creatine lacks this activity and the crystallized impurity is highly active. It has recently been established that the active principle is 1,1-dimethylbiguanide.[4] Prior to characterization of the impurity, a guanidine derivative was implicated through color reactions on thin-layer chromatographs. Consequently, a number of derivatives, including the dimethylbiguanide, had been tested and found to have the glycogen-lowering activity before the nature of the impurity in the Practical Grade creatine was known.

PROCEDURES

Animals. Male rats of the Sprague-Dawley strain weighing 150 to 200 g were used.

Diets and feeding. At the start of an experiment, animals were removed from the stock ration [5] and fed the semipurified diet [6] for 1 day to accustom them to such rations. Some of the rats were maintained on this diet and others were fed this diet supplemented with one of the creatine samples, or a guanidine derivative, or both for an additional 20 hours. At this time each animal that had consumed the standard amount of food was given the final meal in a water slurry by stomach tube as described previously (2).

Received for publication August 11, 1969.

[1] This investigation was supported in part by the Medical Research Foundation of Oregon, Grant 685, and by Public Health Service Research Grant no. AM-04336 from the National Institute of Arthritis and Metabolic Diseases.

[2] Presented in part at the Northwest Regional Meeting of the American Chemical Society, Salt Lake City, June 12–13, 1969, 24th Annual Northwest Meeting, Abstract no. 57.

[3] Through the cooperation of Eastman Organic Chemicals Department, Rochester, N.Y., it was ascertained that the original Practical Grade creatine employed was from Lot No. 12 (Cat No. P951) obtained by Eastman from a European supplier. The later samples lacking the glycogen-lowering activity, were from Lot No. 13, supplied to Eastman by a U.S. firm. Eastman was unable to supply any more of the Lot 12 compound and our supply was exhausted. A note in *Science* requesting Eastman samples purchased by investigators during the time Lot 12 was available brought a heartening response from laboratories in the U.S. and other countries. A number of these samples were tested and showed the same activity as our original supply of Lot 12.

[4] The characterization of the crystalline impurity was kindly carried out by Dr. Doyle Daves, Jr., Oregon Graduate Center, Portland, Oregon 97225.

[5] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Missouri.

[6] Containing: (in percent) casein, 16; salt mixture W, 5 (Nutritional Biochemicals Corp., Cleveland); Squibb flavored yeast, 10 (E. R. Squibb and Sons, New York); cod-liver oil, 2; cottonseed oil, 5; white corn dextrin, 54; glucose, 8.

This was done to equalize food intake and to control the time of food intake prior to the final 4-hour period before the animals were killed. Total food for the 20 hours, including that given by gavage, amounted to 18 to 19 g per rat throughout.

Tissue handling. The animals were anesthetized with pentobarbital sodium [7] given intraperitoneally (8 mg per 200 g body weight). The liver was quickly removed, blotted and minced by one pass through a custom-built "garlic press." An aliquot was added to a tared centrifuge tube containing 30% KOH. The procedure was accomplished in less than 1 minute after opening the abdomen.

Methods. Glycogen was isolated by the method of Good et al. (3), except that the isolated glycogen was dissolved in water and reprecipitated with alcohol. Liberated glucose was estimated by the enzymatic procedure of Salomon and Johnson (4).

The active impurity was isolated from the Practical Grade creatine as follows: creatine was dissolved in a minimum quantity of water at room temperature and precipitated by the addition of 3 volumes of acetone. The filtered supernatant was evaporated to dryness at room temperature aided by a stream of air. The remaining yellowish precipitate (mainly the impurity) was subjected to a similar recrystallization and the solid taken up in water. This was treated with activated carbon, filtered and evaporated as before. The small amount of white crystalline material was not pure but is composed principally of 1,1-dimethylbiguanide [8] and is referred to as crystalline impurity in table 1.

The precipitated creatine was recrystallized two more times in a similar manner and is referred to as "purified" creatine in table 1.

TABLE 1

Liver glycogen levels in rats after 20 hours on the basal semipurified diet with supplements as noted

Dietary supplement	Liver glycogen
	% wet wt
None	4.1 ±0.13 [1](14) [2]
1% Practical creatine [3]	1.1 ±0.09 (17)
1% "Purified" creatine [4]	3.7 ±0.20 (7)
1% "Inactive" creatine [5]	4.5 ±0.17 (6)
0.02% Crystalline impurity	0.8 ±0.18 (10)
1% "Inactive" creatine + 0.02% crystalline impurity	0.7 ±0.11 (9)
0.01% 1,1-Dimethylbiguanide hydrochloride	0.6 ±0.06 (6)
1% "Purified" creatine + 0.01% dimethylbiguanide	0.5 ±0.06 (6)
0.01% Dimethylbiguanide	0.6 ±0.06 (6)
0.01% Galegine [6]	0.4 ±0.07 (6)
0.01% Synthalin [7]	1.0 ±0.12 (9)
0.01% Agmatine [8]	0.9 ±0.11 (6)
0.01% *N*-Butylbiguanide sulfate	0.8 ±0.14 (9)
0.03% Guanidine hydrochloride	2.3 ±0.17 (9)
0.05% Biguanide sulfate	2.2 ±0.16 (9)
0.02% Phenethylbiguanide	2.3 ±0.21 (16)
0.05% Phenethylbiguanide	2.2 ±0.15 (9)

[1] Mean ± SEM.
[2] No. of animals.
[3] Lot 12-Eastman creatine.
[4] Lot 12 creatine, purified by recrystallization from aqueous solution by acetone precipitation (three times).
[5] Lot 13-Eastman creatine.
[6] 4-Methyl-3-butenylguanidine (as sulfate).
[7] Decamethylene diguanidine (as carbonate).
[8] 1-Amino-4-guanidobutane (as sulfate).

RESULTS AND DISCUSSION

It is now established that creatine ingestion does not lower liver glycogen levels in rats as reported earlier from this laboratory (1). An impurity in the creatine originally employed [9] was responsible for this decrease. It was separated, crystallized and characterized as 1,1-dimethylbiguanide.[10] After 20 hours on the semipurified ration containing 0.02% of the crystalline impurity, rats had liver glycogen levels of less than 1% compared with about 4% for animals on the unsupplemented diet (table 1). The findings were similar in animals ingesting authentic 1,1-methylbiguanide as part of the diet. The original "active" creatine [11] lost most of this activity after three recrystallizations, and samples of creatine purchased at a later date [12] (inactive creatine) were without response on liver glycogen levels when ingested by rats under the conditions employed. Rats consuming the biguanide-supplemented diet containing, in addition, either "pure" (inactive) creatine or the original creatine after it had been recrystallized, showed liver glycogen values

[7] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.
[8] See footnote 4.
[9] See footnote 3.
[10] See footnote 4.
[11] See footnote 3.
[12] See footnote 3.

similar to those on the diet without added creatine.

Other guanidine derivatives were found to have a similar physiological action under the conditions outlined. Galegine (4-methyl-3-butenylguanidine), synthalin (decamethylene diguanidine), agmatine (1-amino-4-guanidobutane) and *N*-butylbiguanide salts showed high activity when consumed by rats at a level of 0.01% of the diet. Phenethylbiguanide, guanidine and biguanide salts were far less active even with increased intake (table 1).

Interperitoneal injection of dimethylbiguanide was found to be highly effective in lowering liver glycogen in rats. Animals were injected with 1 ml of bicarbonate buffer, pH 7.4, or this solution containing 2.5 mg of the drug per milliliter following 20 hours feeding and a final meal by gavage as described under Procedures. After 4 hours or 8 hours, liver glycogen levels averaged close to 1% for the drug-injected animals and more than 4% for those given the buffer only.

A number of biguanides are hypoglycemic agents and have been used in the oral treatment of diabetes in adults. The antigluconeogenic activity of several derivatives has been well known for some time. Jangaard and co-workers (5) reported that the ability of dimethylbiguanide, phenethylbiguanide and butylbiguanide to inhibit gluconeogenesis in minced guinea pig liver correlated well with the ability of the drugs to suppress pyruvate oxidation. Oxidations were said to be inhibited at drug levels near those reached in tissues of patients consuming these compounds. Meyer and others (6) demonstrated marked inhibition of hydrocortisone-induced gluconeogenesis in 24-hour fasted rats as a result of pretreating the animals with dimethylbiguanide (20 mg per 200 g body weight). Guinea pigs responded similarly to phenethylbiguanide administered before the hydrocortisone injection. Glucose formation from administered pyruvate or alanine was inhibited by both drugs.

In animals consuming 19 g of ration containing 0.01% dimethylbiguanide as in the present experiments, the drug intake amounts to about 2 mg per rat over the 20-hour feeding period or about one-tenth the amount administered to a 200 g rat in one dose by Meyer et al. (6).

The results presented in the present paper indicate a marked inhibitory effect of ingested guanidine derivatives on gluconeogenesis in rats. This is in accord with the findings of other workers. It is of interest to note that guanidines, diguanidines and biguanides are active but to varying degrees.

LITERATURE CITED

1. Todd, W. R., M. Harris and L. Laastuen 1964 Effects of dietary creatine and related compounds on tissue glycogen deposition in rats. J. Nutr., *82:* 301.
2. Todd, W. R., and M. Allen 1958 Synthesis of tissue glycogen in rats prefed diets with added glycine. Amer. J. Physiol., *194:* 338.
3. Good, C. A., H. Kramer and M. Somogyi 1933 The determination of glycogen. J. Biol. Chem., *100:* 485.
4. Salomon, L. L., and J. E. Johnson 1959 Enzymatic microdetermination of glucose in blood and urine. Anal. Chem., *31:* 453.
5. Jangaard, N. A., J. N. Pereira and R. Pinson 1968 Metabolic effects of the biguanides and possible mechanism of action. Diabetes, *17:* 96.
6. Meyer, F., M. Ipaktchi and H. Clauser 1967 Specific inhibition of gluconeogenesis by biguanides. Nature, *213:* 203.

Differential Requirement for de novo RNA Synthesis in the Starved-refed Rat; Inhibition of the Overshoot by 8-Azaguanine after Refeeding [1]

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ABSTRACT The activities of phosphohexose isomerase, pyruvate kinase, L-α-glycerophosphate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme, dihydroxyacetone kinase and hexose phosphorylating capacity were measured in ad libitum-fed rats, starved rats, and starved rats refed for 1, 2 and 3 days. The activities of all enzymes measured increased to about twice the level measured in rats adapted to ad libitum feeding on days 2 and 3 of refeeding, except the activity of L-α-glycerophosphate dehydrogenase and the hexose phosphorylating capacity, which after the overshoot at 2 days returned to normal levels 3 days after refeeding. Treatment with 8-azaguanine resulted in high levels of liver glycogen and prevented the overshoot in enzyme activities — but not the increase back to normal levels. Phosphorylase activity was the same in both treated and nontreated rats; thus, increased liver glycogen levels in rats treated with azaguanine could not be explained on the basis of phosphorylase activity. 8-Azaguanine did decrease food intake; however, the overshoot was also observed in pair-fed rats. Therefore, the absence of overshoot in azaguanine-treated rats was not due to a decrease in food intake. The observed overshoot in enzyme activities on day 2 of refeeding was not observed in rats which received the first injection of the antibiotic 12 hours after refeeding. A possible explanation of the data is that the overshoot observed after refeeding is dependent on de novo RNA synthesis which occurs at least 12 hours after refeeding.

The activities of a number of rat liver enzymes can be altered by dietary manipulations. These manipulations include changing the amount of dietary protein (1–10), the type of carbohydrates fed (11–14), starvation (15–22) and starvation followed by refeeding (17, 19, 20, 22, 23). Some of the changes mediated by diets occur within a short period after the dietary stimulus — such as the changes in the activities of tryptophan pyrrolase (10), tyrosine-α-ketoglutarate transaminase (4, 9), phosphoenolypyruvate carboxykinase (24), glucokinase (23, 24) and pyruvate kinase (25) — whereas the activities of a number of other enzymes require a period of 2 to 4 days to show maximum change. The control of these changes and the requirement for de novo protein and RNA synthesis have been studied in a number of cases using puromycin (26–28), cycloheximide (29), actinomycin D (28) and ethionine (30). These substances, however, are very toxic even in small doses which limits their use to instances where changes in enzyme activity occur within hours after the administration of an inducing stimulus. In addition, there is good evidence that ethionine (30), cycloheximide and actinomycin D (31) greatly reduce food intake which precludes the use of these antibiotics in dietary studies of longer duration. We have, therefore, decided to search for an inhibitor of protein synthesis, either direct or indirect, which would not greatly affect food intake and thus be suitable in dietary studies of longer duration. From previous work by Kvam and Parks (32, 33), as well as from preliminary work in our laboratory, it appeared that 8-azaguanine might be just such an inhibitor.

8-Azaguanine has been found to inhibit protein synthesis in microorganisms (34, 35) and HeLa cells (36), but not in rabbit reticulocytes (36). It appears that the antibiotic is incorporated into messenger RNA as well as ribosomal RNA (36, 37), which would be expected to cause an alteration in the amino acid sequence of pro-

Received for publication July 1, 1969.
[1] Supported in part by Public Health Service Research Grant no. AM-04732 from the National Institute of Arthritis and Metabolic Diseases.

tein molecules formed (if such RNA can be translated) or a decrease in the capacity of ribosomes to synthesize protein.

We chose the Tepperman experiment (30), refeeding of rats previously starved for 48 hours, as a model system of enzyme induction for two reasons: 1) the activities of a number of enzymes are increased from subnormal to supernormal levels after refeeding, and 2) this overshoot in enzyme activity is essentially maximal 48 hours after refeeding, which is a longer time-period than the short-term dietary effects, but shorter than many other dietary effects.

The use of 8-azaguanine might allow distinction among three possible controls of the starve-refeed response. The first of these possibilities is that the 48-hour starvation causes no major change in existing RNA levels and that the decrease in enzyme activity during starvation and the subsequent overshoot in enzyme activities after refeeding are regulated at the translational level — hence not requiring de novo RNA synthesis. If such is the case, administration of the antibiotic should not abolish the overshoot. In the second possibility, if the overshoot did require de novo RNA synthesis, this response should be inhibited by 8-azaguanine. The third possibility is that a 48-hour starvation does cause a major decrease in RNA. In this case the antibiotic should inhibit not only the overshoot response, but also the recovery of normal enzyme activity. Concurrent with the latter two possibilities is another possibility, namely, that 8-azaguanine might decrease or abolish the starve-refeed response by decreasing food intake. This possibility can be confirmed or eliminated by comparing pair-fed untreated animals to rats treated with the antibiotic. To distinguish among these possibilities the following experiments were undertaken.

EXPERIMENTAL

Treatment of animals. Male rats of the Sprague-Dawley strain weighing 150 to 180 g were fed commercial rat pellets[2] for 3 to 4 days to acclimate them. Rats which were used to establish normal values of enzyme activity under the conditions employed were fed, after having been acclimated, a diet consisting of 65% glucose, 25% casein, 5% corn oil, 4% P.H. salt (38) and 1% vitamins (39). These animals were fed for 5 days before they were killed.

Following acclimatization, groups of rats were starved for 48 hours and then refed with the 65% glucose diet described above. Measurements of body weight and food intake were carried out between 9 and 10 AM.

Unless otherwise indicated, 8-azaguanine was administered intraperitoneally, in two equal doses daily, the first dose at 9 AM and the second dose at 9 PM. The antibiotic was dissolved in dilute sodium hydroxide and the pH of the solution was adjusted to 10. Each dose was administered in 0.5 ml of the solution. Rats which were starved for 48 hours before refeeding were injected with the first dose of the antibiotic just before refeeding and every 12 hours thereafter until killed at 1, 2 and 3 days after refeeding began. The antibiotic was administered at three different dose levels: 7.5 mg per day, 15 mg per day and 30 mg per day. To test whether the presumed increase in de novo RNA synthesis occurs within a short time after refeeding or later, groups of rats were given the first dose of the antibiotic 1 hour prior to refeeding, simultaneously with refeeding and 12 hours after refeeding. These rats were injected with 15 mg 8-azaguanine per day (in two separate doses as described previously), except the group which received its first dose of the antibiotic 12 hours after refeeding: this group received only one dose, or 7.5 mg of the antibiotic during day 1 of refeeding, but was treated with two doses or 15 mg during day 2 of refeeding. The rats were then killed 2 days after initiation of refeeding. The effect of the antibiotic was also tested in rats adapted to ad libitium feeding. This was done by injecting rats with two doses of the antibiotic (one at 9 AM and the second at 9 PM) at each dose level. These rats were killed 24 hours after the first injection. Two sets of controls of the starve-refeed experiments were carried out: one group which received no injections, and another group

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

which was injected twice daily with 0.5 ml per dose of 0.9% NaCl, pH 10.

Food intake and changes in body size were expressed as percentages of body weight at the beginning of the time period under consideration. Pair-fed rats were fed an amount of food consumed by azaguanine-treated animals as calculated on the basis of body weight.

Assay procedures. Rats were killed between 9 and 10 AM by a sharp blow to the head and decapitation, except two groups of rats which were anesthetized with 6 mg of sodium pentobarbital per 100 g body weight and decapitated 20 minutes later. The carcasses were exsanguinated and livers were quickly removed, blotted, weighed and chilled over ice. Liver glycogen was determined by a nephelometric method (40).

A 10% liver homogenate was prepared with ice-cold 0.14 M KCl, pH 7.4, using a Potter-Elvehjem homogenizer. The liver homogenate was centrifuged in the cold at 20,000 × *g* for 30 minutes and the resultant clear supernatant solution was used for the determination of soluble liver protein (41), phosphohexose isomerase (21), pyruvate kinase [3] (42), L-α-glycerophosphate dehydrogenase (21), glucose 6-phosphate dehydrogenase (21), 6-phosphogluconate dehydrogenase (21), malic enzyme (21), dihydroxyacetone kinase [4] and hexose phosphorylating capacity (including both hexokinase and glucokinase).[5] The activities of these enzymes were estimated by determining the amount of NADPH formed or NADH consumed at 25°. Phosphorylase activity was determined by measuring the amount of inorganic phosphate liberated at 37° using a 5% whole liver homogenate prepared with cold, 0.1 M potassium citrate buffer, pH 6.5 (21).

Enzyme activity is reported as units per 100 g body weight. One unit of enzyme activity was defined as that amount of enzyme which produced 1 μmole of measured product under the conditions of the enzyme assay.

RESULTS

Changes in body size and food intake. Rats lost an average of 22% of their body weight during 48 hours of starvation. After refeeding, the weight gains on days 1, 2 and 3 were 13, 6 and 5%, respectively, of body weight. In azaguanine-treated rats the corresponding figures were 10 to 12, 2 to 4, and 4%. Food intakes during the same periods were 12, 13 and 10% of body weight in nontreated rats and 10, 11 and 9% of body weight in azaguanine-treated rats. Food intake and gain in body weight were slightly reduced by the antibiotic. Small reductions were also noted in the animals injected with the saline solution (pH 10). In the groups adapted to ad libitum feeding the daily food intake averaged about 10% of the body weight and the average daily weight gain 3 to 4%. Neither of these values was appreciably reduced by two doses of the antibiotic. It should be noted, therefore, that although the antibiotic did cause a reduction in food intake in the starved-refed animals, food intake in these animals was still at or near the level consumed by rats adapted to ad libitum feeding, which in the case of a 150-g rat, consuming an amount of food equal to 10% of its body weight, amounts to 15 g of food per day.

Effect of refeeding and 8-azaguanine on liver glycogen and soluble protein. In starved animals, liver glycogen was depleted below the level of detection (table 1). Following refeeding the amount of liver glycogen rose to about twice the level measured in rats adapted to ad libitum feeding and fell to normal or nearly normal values on days 2 and 3 of refeeding. In rats treated with 8-azaguanine, the level of liver glycogen remained above normal even on days 2 and 3 of refeeding. This is in sharp contrast to the action of puromycin (43),[6] actinomycin D and cycloheximide (25), all of which tend to decrease liver glycogen. Since it has been shown that liver phosphorylase is decreased by starvation (21), it appeared possible that the antibiotic may prevent the recovery of phosphorylase activity after refeeding, which would lead to high levels of liver glycogen.

[3] The method was modified by increasing the final concentration of phosphoenolpyruvate in the assay mixture from 7.8×10^{-4} M to 3×10^{-3} M.

[4] Murad, S., Doctoral Dissertation. University of California at Davis, 1968.

[5] See footnote 4.

[6] Weber, G., and R. L. Singhal 1963 Puromycin inhibition of cortisone-induced synthesis of hepatic gluconeogenic enzymes. Federation Proc., 22: 636 (abstract).

TABLE 1

Effect of feeding schedule and 8-azaguanine on soluble liver protein and liver glycogen

Treatment	Injections	Soluble liver protein	Liver glycogen
		mg/100 g body wt	
Starved	None	432 [1] ± 29 [2]	< 10
Starved, refed 1 day	None	548 ± 27	405 ± 68
	7.5 mg 8AG [3]	476 ± 23	552 ± 61
	15 mg 8AG	508 ± 19	435 ± 36
	30 mg 8AG	436 ± 37	570 ± 46
	None (pair-fed) [4]	518 ± 19	391 ± 45
Starved, refed 2 days	None	570 ± 23	130 ± 13
	7.5 mg 8AG	527 ± 13	446 ± 56
	15 mg 8AG	530 ± 16	421 ± 32
	30 mg 8AG	574 ± 30	503 ± 47
	PSP (pH 10)	431 ± 21	191 ± 33
	None (pair-fed) [4]	613 ± 38	185 ± 68
Starved, refed 3 days	None	537 ± 12	76.5 ± 11
	15 mg 8AG	548 ± 68	304 ± 10
Adapted to ad libitum feeding	None	546 ± 30	144 ± 21
	7.5 mg 8AG	451 ± 45	189 ± 39
	15 mg 8AG	494 ± 29	147 ± 11
	30 mg 8AG	523 ± 40	189 ± 29
	None (pair-fed) [5]	616 ± 63	208 ± 80

[1] Numbers represent the average of 6 to 12 animals.
[2] SEM.
[3] Abbreviations used: 8AG = 8-azaguanine, PSP = physiological saline (0.9% NaCl, pH 10). 8-Azaguanine was injected intraperitoneally in two equal doses, the first dose at 9 AM and the second dose at 9 PM. Injections were given in 0.5 ml.
[4] These animals were pair-fed to the group injected with 30 mg 8AG/day.
[5] These animals were pair-fed to the group adapted to ad libitum feeding and injected with 30 mg 8AG/day.

We decided to test this hypothesis. Accordingly, the activity of liver phosphorylase was measured in rats refed for 2 days and anesthetized for 20 minutes before killing, and also in rats refed for 3 days and killed without anesthesia. It has been shown that the method of killing influences the measurable activity of phosphorylase (44). In particular, normal methods of decapitation without anesthesia cause sudden shock and activation of phosphorylase B so as to obscure the in vivo status of phosphorylase (44). Since it was of interest to show whether high levels of liver glycogen were a consequence of low phosphorylase activity in vivo, the activity of phosphorylase was determined in anesthetized rats. It was also of interest to see if the recovery of phosphorylase activity after refeeding is inhibited by the antibiotic. In rats refed for 2 days and anesthetized before killing, the activity of liver phosphorylase was 11.8 units/100 g body weight in nontreated and 14.8 units/100 g body weight in azaguanine-treated rats. On day 3 of refeeding, the activity of phosphorylase was 27 units/100 g body weight in both treated and nontreated rats. The results indicate that the high level of liver glycogen in azaguanine-treated rats is not due to a lower level of phosphorylase activity and that the antibiotic did not prevent the recovery of phosphorylase activity after refeeding.

Soluble liver-protein values were 432 mg/100 g body weight in starved rats and 540 to 570 mg/100 g body weight in refed rats. This figure was not increased after days 2 and 3 of refeeding and was reduced by azaguanine treatment only on day 1 after refeeding but not on subsequent days. In rats adapted to ad libitum feeding the level of soluble protein was found to be 530 mg/100 g body weight, and this level was not appreciably altered by azaguanine treatment.

Effect of starvation and refeeding on rat liver enzyme activities. The response of

rat liver enzyme activities to starvation and refeeding is summarized in table 2. The general tendency was a decrease in activities in starved rats, a return to normal or near normal levels after 1 day of refeeding and an overshoot of normal activity on days 2 and 3 of refeeding. The activities of L-α-glycerophosphate dehydrogenase and glucokinase plus hexokinase returned to normal levels on day 3 of refeeding, although these enzymes were increased in activity to levels above normal on day 2 of refeeding.

Rats which were injected with the 0.9% NaCl solution (pH 10), or which were pair-fed to the azaguanine-treated group consuming the smallest amount of food (i.e., those given 30 mg 8-azaguanine/day), responded to refeeding in nearly the same way as did the rats refed ad libitum and not receiving any injections. The effect of azaguanine treatment, therefore, cannot be attributed to a reduction in food intake or the injection of an alkali solution. Although the extent of overshoot of L-α-glycerophosphate dehydrogenase activity was reduced by pair-feeding on days 1 and 2 of refeeding, the overshoot was still observable.

Effect of 8-azaguanine on the recovery of enzyme activity after refeeding and in rats adapted to ad libitum feeding. It should be noted that, since azaguanine treatment did not result in smaller values of relative liver size or soluble liver protein, the effects of the antibiotic on protein synthesis may have been specific and limited to those enzymes which required the greatest amount of de novo RNA synthesis. It is also possible that the antibiotic may have caused the production of proteins in which the amino acid sequence had been altered causing a reduction in enzyme activity. Although this possibility cannot be ruled out, it is not likely that RNA containing 8-azaguanine could function normally with respect to rate of amino acid incorporation or binding of ribosomes. In fact, previous work indicates that RNA containing 8-azaguanine may be nonfunctional (37).

The effects of 8-azaguanine on the activities of several liver enzymes in ad libitum-fed rats and in starved-refed rats is summarized in table 3. It is apparent that the antibiotic inhibited the overshoot in enzyme activity on days 2 and 3 of refeeding. It is also noteworthy that enzyme activities returned to nearly normal after refeeding for 2 days, even in the antibiotic-treated rats, which indicates that it is the overshoot response which may be dependent on de novo RNA synthesis. However, the recovery of pyruvate kinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities was less than the recovery of other enzymes after refeeding. From this it can be concluded that a 48-hour starvation apparently does not cause a major decrease in the RNA component of the synthetic apparatus, although it is possible that there was some damage, especially in the case of the latter three enzymes. The question arose, therefore, whether the de novo RNA synthesis necessary for the overshoot is accomplished within the first few hours of refeeding or later. Accordingly, three groups of rats starved for 48 hours and then refed were treated with 8-azaguanine 1 hour before refeeding, simultaneously with refeeding and 12 hours after refeeding; the treatment was then repeated at 12-hour intervals until the rats were killed 48 hours after refeeding. The results are summarized in table 4. The overshoot in enzyme activity was not detectable even in the group which was first injected 12 hours after refeeding. The data suggest, therefore, that the bulk of the de novo RNA synthesis, which appears to be required for the overshoot response, takes place at least 12 hours after refeeding is initiated.

DISCUSSION

We have set out to find an antibiotic which would be useful in dietary studies of longer duration. One major requirement of such usefulness is that the antibiotic would not decrease food intake to a level which would no longer produce the metabolic response associated with that particular diet. It appears that 8-azaguanine may fill this requirement, since even at very high doses of the antibiotic, food intake was not greatly reduced; moreover, the metabolic response could also be shown if food intake was limited to that consumed by the rats treated with the antibiotic.

TABLE 2

Effect of starvation and refeeding on rat liver enzyme activities

Treatment	Normal [1]	Starved	Refed 1 day		Refed 2 days			Refed 3 days
			Ad libitum	Pair-fed [2]	Ad libitum	Pair-fed [2]	Ad libitum + PSP [3]	Ad libitum
Relative liver size [4]	4.89 [5] ± 0.17 [6]	3.44 ± 0.11	6.13 ± 0.16	5.96 ± 0.19	5.98 ± 0.20	5.35 ± 0.20	5.25 ± 0.31	5.55 ± 0.12
Phosphohexose isomerase	363 [7] ± 23	207 ± 14	296 ± 6.2	391 ± 45	556 ± 28	534 ± 47	513 ± 42	624 ± 22
Pyruvate kinase	106 ± 15	14.7 ± 2.2	63.1 ± 3.8	85.0 ± 10	152 ± 13	149 ± 11	145 ± 14	163 ± 6.4
L-α-Glycerophosphate dehydrogenase	148 ± 8.7	49.6 ± 2.2	148 ± 7.9	87.7 ± 12	315 ± 27	210 ± 22	247 ± 24	138 ± 17
Glucose-6-phosphate dehydrogenase	29.5 ± 6.0	3.54 ± 0.59	28.1 ± 2.0	28.2 ± 3.9	77.8 ± 7.0	86.2 ± 9.3	67.8 ± 6.1	86.9 ± 8.0
6-Phosphogluconate dehydrogenase	18.0 ± 1.3	6.69 ± 0.42	7.23 ± 0.46	16.1 ± 1.5	25.6 ± 0.86	35.5 ± 2.9	24.8 ± 1.4	29.9 ± 1.1
Malic enzyme	12.5 ± 2.1	7.16 ± 0.98	19.8 ± 1.1	11.1 ± 2.6	28.3 ± 1.6	26.5 ± 1.6	32.2 ± 4.4	32.5 ± 2.3
Dihydroxyacetone kinase	10.1 ± 0.95	4.77 ± 0.52	7.75 ± 0.43	7.88 ± 0.55	13.5 ± 1.1	14.1 ± 1.2	13.3 ± 0.66	14.6 ± 0.60
Hexose phosphorylating capacity (hexokinase + glucokinase)	10.7 ± 0.92	1.78 ± 0.23	5.00 ± 0.61	11.7 ± 0.94	21.6 ± 1.4	21.0 ± 2.8	[8]	11.9 ± 1.2

[1] Adapted to ad libitum feeding. These animals are referred to as normal.
[2] Pair-fed to the group given 30 mg 8AG/day; fed food, equal to 9.7% of body weight on day 1 and 9.0% of body weight on day 2 of refeeding.
[3] Injected twice daily (9 AM and 9 PM) with 0.5 ml 0.9% NaCl, pH 10.
[4] (Liver wt × 100)/(body weight).
[5] Numbers represent the average of 6 to 12 animals.
[6] SEM.
[7] Enzyme activity is given in units per 100 g body weight. One unit of enzyme activity was defined as that amount of enzyme which can produce 1 μmole of measured product per minute under the conditions of the assay.
[8] Values not determined.

TABLE 3

Effect of 8-azaguanine on rat liver enzyme activities

	Normal [1]	Normal [1]			Refed 1 day			Refed 2 days			Refed 3 days
Injections, mg 8AG/day	0	7.5	15	30	7.5	15	30	7.5	15	30	15
Relative liver size [2]	4.89 ± 0.17 [3]	4.81 ± 0.19	4.45 ± 0.20	4.72 ± 0.12	5.90 ± 0.28	6.04 ± 0.11	6.02 ± 0.25	5.90 ± 0.29	6.29 ± 0.18	6.29 ± 0.23	5.60 ± 0.23
Phosphohexose isomerase	363 [4] ± 23	301 ± 26	282 ± 19	308 ± 6.9	252 ± 39 (85) [5]	187 ± 7.8 (63)	232 ± 16 (79)	439 [a] ± 25 (79)	371 [a] ± 33 (67)	418 [a] ± 23 (75)	516 [a] ± 28 (83)
Pyruvate kinase	106 ± 15	95.5 ± 17	70.7 ± 6.8	88.0 ± 6.9	27.4 ± 3.7 (43)	27.5 ± 3.8 (43)	30.2 ± 3.9 (48)	72.9 [a] ± 23 (48)	62.1 [a] ± 9.5 (42)	68.1 [a] ± 4.9 (45)	60.9 [a] ± 6.9 (32)
L-α-Glycerophosphate dehydrogenase	148 ± 8.7	78.1 ± 6.3	106 ± 16	87.1 ± 4.2	63.3 ± 5.4 (43)	120 ± 7.7 (81)	80.0 ± 8.4 (54)	129 [a] ± 14 (41)	208 [a] ± 17 (66)	109 [a] ± 18 (35)	110 [b] ± 24 (80)
Glucose 6-phosphate dehydrogenase	29.5 ± 6.0	24.9 ± 4.3	19.2 ± 3.3	14.2 ± 1.1	10.3 ± 1.1 (37)	8.32 ± 0.59 (30)	8.95 ± 0.67 (32)	26.2 [a] ± 2.8 (34)	21.6 [a] ± 3.6 (28)	17.9 [a] ± 2.3 (23)	16.7 [a] ± 3.6 (19)
6-Phosphogluconate dehydrogenase	18.0 ± 1.3	16.2 ± 1.6	14.3 ± 1.1	11.5 ± 1.1	9.70 ± 0.49 (134)	3.73 ± 0.26 (52)	10.8 ± 0.63 (149)	15.4 [a] ± 0.92 (60)	14.4 [a] ± 0.82 (56)	13.5 [a] ± 1.6 (53)	11.0 [a] ± 1.4 (37)
Malic enzyme	12.5 ± 2.1	10.6 ± 0.15	10.9 ± 2.1	6.99 ± 0.85	6.77 ± 0.28 (34)	5.09 ± 0.94 (26)	6.74 ± 0.92 (34)	13.9 [a] ± 1.2 (49)	20.6 [a] ± 3.0 (73)	10.8 [a] ± 1.6 (38)	9.18 [a] ± 1.1 (28)
Dihydroxyacetone kinase	10.1 ± 0.95	8.75 ± 0.89	6.31 ± 0.62	7.74 ± 0.45	5.95 ± 0.82 (77)	5.90 ± 0.44 (76)	5.01 ± 0.29 (65)	10.4 [b] ± 1.0 (77)	9.99 [a] ± 0.58 (64)	10.2 [a] ± 0.87 (75)	12.1 [b] ± 1.6 (82)
Hexose phosphorylating capacity (glucokinase + hexokinase)	10.7 ± 0.92	11.2 ± 1.5	9.10 ± 0.92	10.2 ± 0.67	8.12 ± 0.95 (163)	3.37 ± 0.25 (61)	7.30 ± 0.63 (146)	12.2 [a] ± 1.1 (67)	13.8 [a] ± 0.43 (64)	12.6 [a] ± 2.8 (58)	10.2 [b] ± 1.3 (86)

[1] Normal rats = rats adapted to ad libitum feeding.
[2] (Liver weight ×100)/(body weight).
[3] SEM. Each mean is the average of 6 to 12 animals.
[4] Enzyme activity is expressed as units per 100 g body weight. One unit of enzyme activity was defined as that amount of enzyme which produces 1 μmole of measured product under the conditions of the assay.
[5] Numbers in parentheses represent enzyme activity on a particular day of refeeding in 8-azaguanine-treated rats as percentage of enzyme activity in rats refed the same length of time but not treated with 8-azaguanine.
[a] Differs significantly ($P < 0.05$) from values in rats not receiving 8-azaguanine.
[b] Does not differ significantly from values in rats not receiving 8-azaguanine.

TABLE 4

Effect of time of injection of 8-azaguanine on the 2-day overshoot in starved-refed rats [1]

Time injections began	1 hour before refeeding	At time of refeeding	12 hours after refeeding
Relative liver size [2]	6.49 [3]	5.14	6.05
Phosphohexose isomerase	95.6	82.6	78.2
Pyruvate kinase	36.0	51.1	49.4
L-α-Glycerophosphate dehydrogenase	47.0	34.3	43.8
Glucose 6-phosphate dehydrogenase	26.4	21.1	26.4
6-Phosphogluconate dehydrogenase	49.3	45.0	61.8
Malic enzyme	32.8	31.0	31.7
Dihydroxyacetone kinase	83.8	69.3	73.0
Hexose phosphorylating capacity (hexokinase + glucokinase)	72.7	53.2	58.8

[1] Enzyme activity is expressed as percentage of starved-refed ad libitum values. The full overshoot, therefore, is defined as 100%. Rats adapted to ad libitum feeding have enzyme activities which fall in the vicinity of 50% on this scale.
[2] (Liver wt × 100)/(body weight).
[3] Numbers represent the average of 4 animals.

As noted above, the liver glycogen values remained in excess of normal in the antibiotic-treated rats and this could not be accounted for on the basis of lowered phosphorylase activity, since no difference between treated and nontreated values could be shown. In addition, the livers of rats starved and refed for 3 days were yellowish and the centrifuged homogenates of such livers were covered by a thick layer of solidified lipids, whereas the livers of azaguanine-treated rats were normal in color and their centrifuged homogenates were covered only by a thin layer of lipid precipitate. These qualitative observations suggest that in azaguanine-treated rats the low level of shunt activity may be accompanied by a low level of lipid deposition. Since liver is primarly a gluconeogenc organ, and since the activities of the hexose monophosphate shunt dehydrogenases were decreased in the livers of rats refed for 3 days and treated with azaguanine, it may be that the high level of liver glycogen found in these rats was a consequence of decreased shunt activity, a decrease in lipid deposition and, hence, an increase in glycogen deposition as an alternate way of storing a potential source of energy. Clarification of this point awaits further experimental evidence.

In the introduction we have outlined three possible alternative mechanisms to explain the starve-refeed response: 1) that starvation does not bring about a major decrease of RNA necessary for protein synthesis and that the observed overshoot after refeeding is due solely to translational control of enzyme synthesis and degradation in which case 8-azaguanine should not abolish the overshoot; 2) that, although starvation does not significantly decrease existing levels of RNA, the overshoot does require de novo RNA synthesis; and 3) that starvation does cause substantial damage to RNA, in which case not only the overshoot, but also the recovery of normal enzyme activity in liver would be dependent on de novo RNA synthesis and hence, would be abolished by azaguanine treatment. The data are most consistent with the second possibility and nearly eliminate the third. A considerable degree of ambiguity still remains, however. It is possible that either messenger RNA or ribosomal RNA has a moderately long half-life, that azaguanine prevents the maintenance of normal levels of RNA and that the effects of possible translational factors are masked by a progressive decrease in available, functional RNA. If such is the case, azaguanine treatment should facilitate the return of enzyme levels to normal after a new steady state of synthesis and degradation has been achieved during the overshoot, provided the overshoot is maintained for a sufficient length of time in order that this hypothesis can be tested. At present, however, the hypothesis that in the starved-refed response the overshoot requires de novo RNA synthesis, whereas restoration of normal en-

zyme activity does not, appears to be the most plausible.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Margaret C. Hill for her technical assistance.

LITERATURE CITED

1. Mandelstam, J., and J. Yudkin 1952 The effect of variation of dietary protein upon the hepatic arginase of the rat. Biochem. J., *51:* 681.
2. Schimke, R. T. 1962 Adaptive characteristics of the urea cycle enzymes in the rat. J. Biol. Chem., *237:* 459.
3. Rosen, F., N. R. Roberts and C. A. Nichol 1959 Glucocorticoids and transaminase activity. I. Increased activity of glutamic-pyruvic transaminase in four conditions associated with gluconeogenesis. J. Biol. Chem., *234:* 476.
4. Freedland, R. A., T. L. Cunliffe and J. G. Zinkl 1966 The effect of insulin on enzyme adaptations to diets and hormones. J. Biol. Chem., *241:* 5448.
5. Waldorf, M. A., and A. E. Harper 1963 Metabolic adaptations in higher animals. VIII. Effect of diet on ornithine-α-ketoglutarate transaminase. Proc. Soc. Exp. Biol. Med., *112:* 955.
6. Muramatsu, K., and K. Ashida 1962 Effect of dietary protein level on growth and liver enzyme activities of rats. J. Nutr., *76:* 143.
7. Waldorf, M. A., M. C. Kirk, H. Linksweiler and A. E. Harper 1963 Metabolic adaptations in higher animals. VII. Responses of glutamate-oxaloacetate and glutamate-pyruvate transaminase to diet. Proc. Soc. Exp. Biol. Med., *112:* 764.
8. Freedland, R. A., and E. H. Avery 1964 Studies on threonine and serine dehydrase. J. Biol. Chem., *239:* 3357.
9. Pitot, H. C., V. R. Potter and H. P. Morris 1961 Metabolic adaptations in rat hepatomas. I. The effect of dietary protein on some inducible enzymes in liver and hepatoma 5123. Cancer Res., *21:* 1001.
10. Rosen F., H. R. Harding, R. J. Milholland and C. A. Nichol 1963 Glucocorticoids and transaminase activity. VI. Comparison of the adaptive increase of alanine- and tyrosine-α-ketoglutarate transaminase. J. Biol. Chem., *238:* 3725.
11. Freedland, R. A., and A. E. Harper 1959 Metabolic adaptations in higher animals. V. The determination of metabolic pathways by the use of metabolic adaptations. J. Biol. Chem., *234:* 1350.
12. Fitch, W. M., and I. L. Chaikoff 1960 Extent and patterns of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., *235:* 554.
13. Krebs, H. A., and L. V. Eggleston 1964 The role of pyruvate kinase in the regulation of gluconeogenesis. Biochem. J., *94:* 3c.
14. Gaertner, E. B., and C. Carroll 1967 Metabolic responses of white rats to balanced and imbalanced protein fed with different carbohydrates in 15% and 5% fat diets. J. Nutr., *91:* 69.
15. Allard, C., G. DeLamirande and A. Cantaro 1957 Behavior of enzymes in liver of starved rats. Exp. Cell Res., *13:* 69.
16. Weber, G., and A. Cantero 1954 Glucose-6-phosphatase studies in fasting. Science, *120:* 857.
17. Niemeyer, H., C. Gonzales and R. Rozzi 1961 Influence of diet on liver phosphorylase. I. Effect of fasting and refeeding. J. Biol. Chem., *236:* 610.
18. Schimke, R. T. 1962 Differential effect of fasting and protein-free diets on levels of urea cycle enzymes in rat liver. J. Biol. Chem., *237:* 1921.
19. Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes, *7:* 478.
20. Niemeyer, H., L. Cark-Turri, E. Garces and F. E. Vergara 1962 Selective response of liver enzymes to the administration of different diets and after fasting. Arch. Biochem. Biophys., *98:* 77.
21. Freedland, R. A. 1967 Effect of progressive starvation on rat liver enzyme activities. J. Nutr., *91:* 489.
22. Adelman, R. C., P. D. Spolter and S. Weinhouse 1966 Dietary and hormonal regulation of enzymes of fructose metabolism in rat liver. J. Biol. Chem., *241:* 5467.
23. McDonald, B. E., and B. C. Johnson 1965 Metabolic response to realimentation following chronic starvation in the adult male rat. J. Nutr., *87:* 161.
24. Lardy, H. A., D. O. Foster and E. Shrago 1964 Metabolic regulation of phosphoenolpyruvate synthesis. Advan. Enzyme Regulat., *2:* 39.
25. Szepesi, B., and R. A. Freedland 1968 Dietary regulation of pyruvate kinase synthesis in rat liver. J. Nutr., *95:* 591.
26. Weber, G., and R. L. Singhal 1964 Role of enzymes in homeostasis. V. Actinomycin and puromycin inhibition of cortisone induced synthesis of hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase. J. Biol. Chem., *239:* 521.
27. Grossman, A., and C. Mavrides 1967 Studies on the regulation of tyrosine aminotransferase in rats. J. Biol. Chem., *242:* 1398.
28. Rowe, P. B., and J. B. Wyngaarden 1966 The mechanism of dietary alterations in rat hepatic xanthine oxidase levels. J. Biol. Chem., *241:* 5571.
29. Kenney, F. T. 1967 Turnover of rat tyrosine transaminase: stabilization after inhibition of protein synthesis. Science, *156:* 525.
30. Tepperman, H. M., and J. Tepperman 1963 On the response of hepatic glucose 6-phosphate dehydrogenase activity to changes in diet composition and food intake pattern. Advan. Enzyme Regulat., *1:* 121.
31. Ray, P. D., D. O. Foster and H. A. Lardy 1964 Mode of action of glucocorticoids. I.

Stimulation of gluconeogenesis independent of synthesis de novo of enzymes. J. Biol. Chem., 239: 3396.
32. Kvam, D. C., and R. E. Parks, Jr. 1960 Hydrocortisone-induced changes in hepatic glucose 6-phosphatase and fructose diphosphatase activities. Amer. J. Physiol., *198:* 21.
33. Kvam, D. C., and R. E. Parks, Jr. 1960 Inhibition of hepatic induced enzyme formation by 8-azaguanine. J. Biol. Chem., *235:* 2893.
34. Mandel, H. G. 1958 Effect of 8-azaguanine on utilization of methionine by B. CEREUS. Arch. Biochem. Biophys., 76: 230.
35. Grünberger, D., L. Messner, A. Holý and F. Šorm 1966 The effect of polymers and trinucleotides containing 8-azaguanine upon the binding of (^{14}C) valine — sRNA to ribosomes. Biochim. Biophys. Acta, *119:* 432.
36. Zimmerman, E. F., and S. A. Greenberg 1965 Inhibition of protein synthesis by 8-azaguanine. I. Effect of polyribosomes in HeLa cells. Mol. Pharmacol., *1:* 113.
37. Kwan, S. W., and T. E. Webb 1967 A study of the mechanism of polyribosome breakdown induced in regenerating liver by 8-azaguanine. J. Biol. Chem., 242: 5542.
38. Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.
39. Benevenga, N. J., W. J. Stileau and R. A. Freedland 1964 Factors affecting the activity of pentose phosphate-metabolizing enzymes in rat liver. J. Nutr., 84: 345.
40. Hansen, R. G., W. J. Rutter and E. M. Craine 1952 A nephelometric method for the determination of glycogen. J. Biol. Chem., *195:* 127.
41. Mokrasch, L. C., and R. W. McGilvery 1956 Purification and properties of fructose-1,6-diphosphatase. J. Biol. Chem., 221: 909.
42. Bucher, T., and G. Pfleiderer 1955 Pyruvate kinase from muscle. In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 435.
43. Hofert, J., J. Gorski, G. C. Mueller and R. K. Boutwell 1962 Depletion of liver glycogen in puromycin-treated animals. Arch. Biochem. Biophys., 97: 134.
44. Hornbrook, K. R., and R. M. Brody 1963 Phosphorylase activity in rat liver and skeletal muscle after catecholamines. Biochem. Pharmacol., *12:* 1407.

Evaluation of Bone Calcium Accretion Rate as a Function of Age in Beagle Dogs [1]

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ABSTRACT In an attempt to determine normal bone growth rate patterns in beagle dogs, bone calcium accretion studies were performed on a group of six animals periodically from age 6 weeks to age 13 months. Accretion rates (A values) were determined by a whole-body counting technique using ^{47}Ca as the tracer. On the basis of the entire skeleton, A values rose from about 1.8 g/day at 6 weeks of age to a maximum of about 3.3 g/day at age 2.5 months. From age 2.5 to 5.0 months, A values remained at a level of 2.8 to 3.0 g/day after which they gradually fell to a low of 0.3 g/day at 13 months of age. Accretion rates for the whole skeleton appeared to follow the growth rate data for beagle dogs. On the basis of unit bone mass, A values decreased from a maximum of 13.80 mg Ca/gram bone per day at 6 weeks to a low of 0.34 mg Ca/g bone per day at age 13 months. The exchangeable calcium-pool size changed roughly in proportion to the A values. The 3-week retention of isotope was greatest at the earliest ages, and decreased with age thereafter.

A few reports have been published showing differing bone growth rates in dogs of differing ages. For example, Jowsey et al. (1), in a study using 3 mongrel dogs aged 9 months, 2.5 years, and 10 years, indicated that the rate of deposition of calcium into bone was greater in the young animal and less in the aged. Lee et al. (2) measured calcium accretion in two dogs, one 3 months old and one about 1 year old and found that the accretion rate was greater in the younger dog. However, no complete study has yet been published regarding the effect of age on bone growth rates in the dog.

There are a number of radiotracer kinetic techniques available for determining the rate of calcium accretion in bone (3–5). Perhaps the best known of these methods is that of Bauer, Carlsson and Lindquist (3). By using a whole-body counter and the radioactive isotope ^{47}Ca, kinetic data can be obtained nondestructively on a whole-skeleton basis which can then be used to determine bone calcium "accretion" by means of the "BCL" formula. Since the half-life of ^{47}Ca is rather short (4.7 days) the same animal can be used again about 1 month after a previous experiment.

The purpose of the studies reported here was to determine the effect of age on calcium accretion rate in a group of normal beagle dogs. The data obtained could then be used as a basis for determining the effect of various abnormal situations, nutritional and otherwise, on bone mineral kinetics in the dog.

MATERIALS AND METHODS

A group of six littermate beagle dogs (four males and two females) was used for these studies. They were maintained from weaning on a standard pelleted dog ration containing 2.1% calcium and 1.4% phosphorus on a dry weight basis.[2] Both food and water were given ad libitum. These puppies were then divided into two groups of three each. Beginning at 6 weeks of age one group of three puppies was used for an accretion study approximately every 2 weeks. Thus each individual dog had his bone calcium accretion rate determined about once a month.

After determining the background count of a puppy in the whole-body counter, the dog was given an intravenous injection of 10 to 20 μCi of $^{47}CaCl_2$ in 1 ml of isotonic sodium chloride solution.[3] A duplicate dose was placed in a water-filled plastic phantom that approximated as closely as possible the geometry of the dog. The radio-

Received for publication July 22, 1969.
[1] Supported by U.S. Atomic Energy Commission contract AT(30-1)-2147.
[2] Big Red Dog Pellets, Agway, Inc., Syracuse, N. Y.
[3] Iso/Serv Division of Cambridge Nuclear Corporation, Boston, Mass.

activity in the dog and in the duplicate dose standard were then immediately counted and the zero-time 100% dose was thus established. The radioactivities in dogs and standard were recounted at 1, 2, 6, and 24 hours and then two or three times weekly for a period of 3 weeks after injection. During the first 24 to 48 hours postinjection, periodic blood samples were taken. The plasma was collected for total and radiocalcium determination.

Radiocalcium was determined in a well-type scintillation counter using pulse height analysis to discriminate against the ^{47}Sc daughter. Total plasma calcium was assayed by atomic absorption spectrophotometry.

The data points for the plasma specific activity curve were entered into an IBM model 1800 computer and a least squares estimate of the equation of the specific activity curve was obtained. The computer was then used to integrate the curve and determine the accretion rate and exchangeable calcium pool size according to the method of Bauer, Carlsson and Lindquist (3).

Initially, accretion rates (A values) were determined at various time intervals after injection of the tracer. It was found that A values remained constant between 24 and 72 hours after injection of tracer. Hence, all values reported here are for the period from 24 to 72 hours postinjection.

For the expression of data on the basis of unit bone mass, an approximation was made of the bone mass of the individual by assuming that 5.35% of body weight is bone (2, 6).

RESULTS AND DISCUSSION

In all cases a 2-term exponential equation gave the best fit to the specific activity data. As shown in figure 1, it was found that the specific activity curve fell more rapidly in young dogs than in the same dogs at older ages. Similar observations have been made by others (1, 2).

The whole-body retention curves of the tracer dose of ^{47}Ca, on the other hand, fell more slowly in young dogs when compared with curves at an older age (fig. 2). That is, the whole-body retention of isotope appeared to be greater in younger dogs. Table

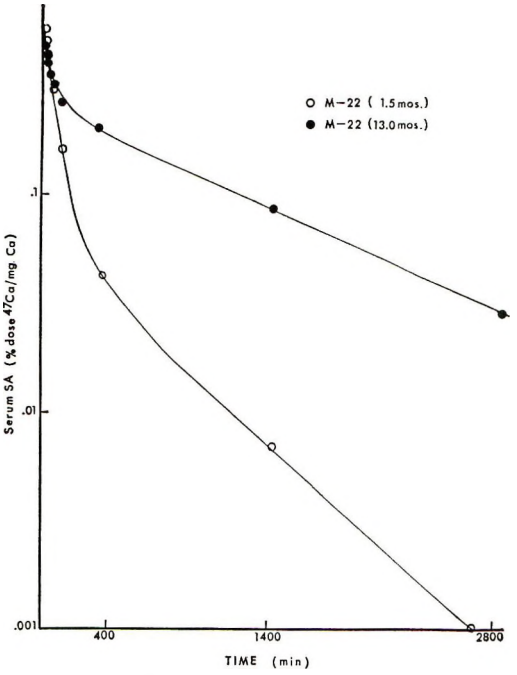

Fig. 1 Serum specific activity curve as a function of time in the same dog at ages 1.5 and 13 months.

1 depicts the 3-week retention of ^{47}Ca as a function of age in all dogs from 6 weeks to 13 months of age, showing that retention fell gradually from a maximum of about 85% at 6 weeks to 41% at 13 months.

Since the specific activity curve fell more rapidly in younger dogs whereas the whole-body retention was greater, it appears that tracer and, by inference, stable calcium was being more rapidly removed from the blood and stored at some site in the body in the young dog compared to older animals. The most likely storage site, of course, is bone.

Accretion rate values of this group of dogs on a whole-skeleton basis are shown in table 1 and figure 3. Regression analysis of the data from 1.5 to 2.5 months of age showed a significant increase in A value from 1.75 g/day at 6 weeks of age to 3.34 g/day at 2.5 months of age ($P < 0.05$). From 2.5 to 5.0 months of age, A values appeared to remain constant at an average value of about 3.00 g/day. During this period accretion rate was independent of age with $P > 0.05$ for a test of inde-

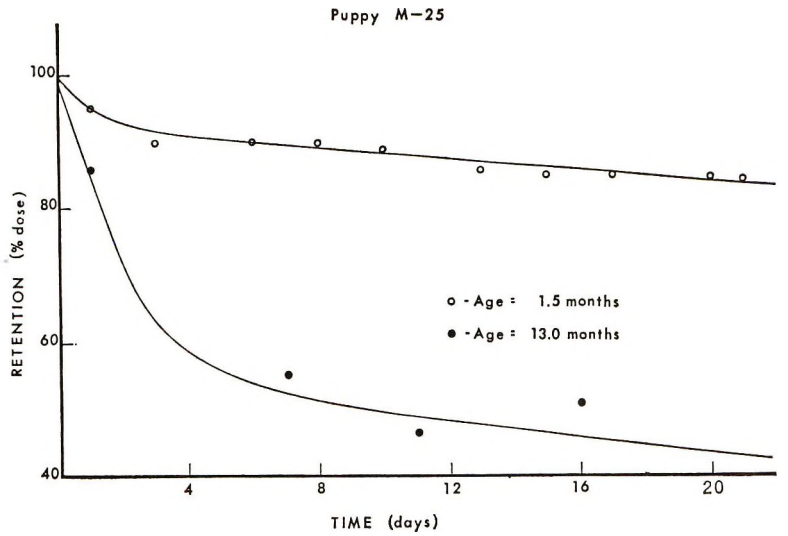

Fig. 2 Whole-body retention of an intravenous dose of ^{47}Ca as a function of time in the same dog at ages 1.5 and 13 months.

TABLE 1

Effect of increasing age on 21-day retention of an intravenous dose of ^{47}Ca, and on total skeletal and unit skeletal accretion rate (A value) and exchangeable calcium (E value) in beagle dogs

Age	Retention 21 days	A value		E value	
		Total skeletal	Unit skeletal	Total skeletal	Unit skeletal
Months	*%*	*g/day*	*mg Ca/g bone per day*	*g*	*mg Ca/g bone*
1.5	85.3	1.752	13.84	1.203	9.48
2.0	80.3	1.795	11.81	1.280	8.40
2.5	79.1	3.336	13.15	1.990	7.71
3.5	80.3	2.851	9.62	1.963	6.70
4.0	84.7	3.000	7.75	2.130	5.52
4.5	85.0	2.789	7.04	1.763	4.44
5.0	79.3	2.880	5.12	1.913	3.41
6.0	79.0	2.357	4.42	2.147	4.05
7.0	73.3	2.083	2.98	2.067	2.98
7.5	71.3	1.699	2.99	1.443	2.54
8.0	75.3	1.749	2.36	1.503	2.07
9.0	60.0	0.908	1.59	1.423	2.48
10.0	65.3	1.384	1.83	1.777	2.35
11.0	54.9	0.809	1.01	1.139	1.43
13.0	41.0	0.285	0.34	0.767	0.91

pendence. After 5 months of age a significant decrease was found in the A values from the high of 3.00 g/day at 5 months of age to a low of 0.29 g/day at 13 months ($P < 0.001$). Thus, on a whole-skeleton basis, A values increased up to about the age of puberty and decreased thereafter. A similar trend has been found for the accretion rate in the human being by Bauer et al. (3). In the human, A values increase from infancy to puberty and decrease thereafter. On a whole body basis the exchangeable calcium pool size (table 1) appeared to follow roughly the same trend as the accretion rate, increasing from a value of 1.20 g at 6 weeks to a maximum of 2.15 g at 6 months of age and then decreasing gradually to a low of 0.77 g at

age 13 months. The changes in the exchangeable calcium with age, however, were more irregular than in the A values.

When the data were expressed on the basis of activity per unit bone mass, a different pattern appeared (table 1). In this case regression analysis demonstrated that the A value decreased significantly from a maximum of about 13.84 mg Ca/g bone per day at 1.5 months to a low of 0.34 mg Ca/g bone per day at 13 months ($P < 0.001$). The exchangeable calcium pool size again appeared to follow roughly the same trend as the accretion rate, dropping from a high of 9.48 mg Ca/g bone at 1.5 months to a low of 0.91 mg/g at 13 months of age.

Considering the two methods of expressing the data it appears that at early ages, while the bone is very active in accumulating calcium, the total amount of bone present is so small that the total skeletal accretion rate is rather low. At puberty, while the unit activity of bone has decreased somewhat, the amount of bone present has increased substantially such that the total accretion rate is relatively high. After puberty, while the amount of bone present stays roughly constant, the unit accretion rate continues to fall and thus the total skeletal accretion rate is reduced.

In other words, the variation in total skeletal accretion rate seen is the result of two processes. The first of these is the increase in total bone mass as a function of age; this plateaus at about 1 year of age. The second process is the decreasing rate of accretion that occurs from the earliest age (table 1). As a result of these two processes, the pattern seen in figure 3 obtains.

Using the data from table 1 it is possible to compare the A values obtained in this study with those of Lee et al. (2). In a 3-month old dog, they observed an accretion rate of 9.0 mg Ca/g bone per day which is in the same range as the 13.8 mg found in this study for beagles of similar age. At greater ages it is difficult to compare our results with those of Lee et al. (2). They report a value of 1.9 mg Ca/g bone per day for a dog estimated at 1 to 2 years of age. In the results reported here the A value at 11 months was 1.0 mg Ca/g bone per day which is in the same range while at 13 months it was only 0.34 mg/gram bone day (table 1). Whether or not this precipitous drop between 11 and 13 months is real remains to be determined. Since the animals used by Lee et al. (2) were reported to be mongrels, breed differences may be responsible for part of the discrepancy. The results reported here, on the other hand, compare quite favorably with those of Green et al. (7) in normal dogs of roughly similar size and age.

It has been argued that the accretion rate includes a number of long-term exchange

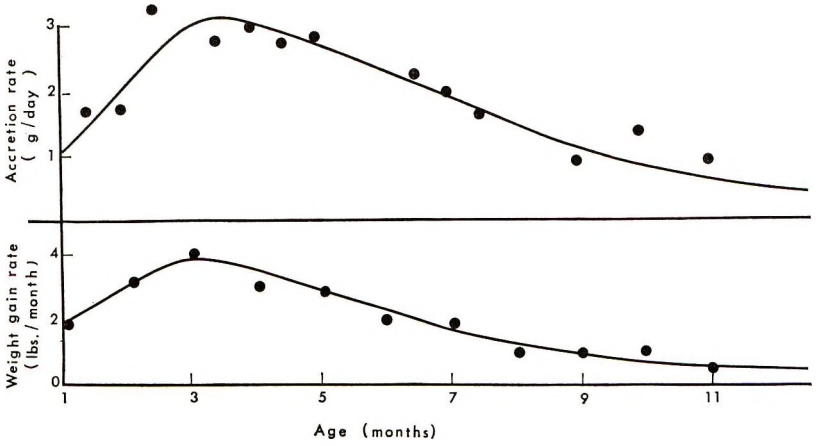

Fig. 3 Effect of age on accretion rate (A value) and weight gain rate in beagle dogs.

components and hence may have little or no physiological significance (8). Although some exchange components may be included, the fact that the A value varies directly with the rate of weight gain (fig. 3) would seem to indicate that the accretion rate does, indeed, have some physiological significance. A similar dependence of the A value on rate of weight gain has been reported by Bauer et al. (3) for man and by Bronner et al.[4] for the rat.

Radiocalcium kinetic studies in adult dogs have been performed by Green et al. (7) under various physiological and pathological conditions. Although there appeared to be quite a bit of normal variability in the sizes of the A value and the exchangeable calcium pool, the values change appreciably under abnormal conditions, e.g., after thyroparathyroidectomy, again indicating the physiological significance of these parameters.

In addition to determination of A values by the isotopic method, the increase in calcium mass of the skeleton was also estimated from increase in body weight. For this calculation it was assumed that fresh bone contains 26% calcium by weight and that 5.35% of body weight is bone (2, 6, 9). Table 2 shows the results of this calculation compared with experimental values obtained from radiocalcium kinetic data in two beagle dogs about 8 weeks of age. In both cases the experimental values are higher than the calculated values by somewhat more than 50%. This is as expected since the experimental approach would provide total accretion while the calculated value would give net accretion, i.e., accretion minus resorption. From the data the resorption rate could be determined and this was found to be from 33 to 43% of the A value. The ratio of accretion rate and resorption rate found here compares favorably with that found for the rat by Bauer et al. (3) and by Wasserman (10).

TABLE 2

Experimental and theoretical estimations of accretion rate (A value) in two dogs

Animal	Theoretical A [1]	Experimental A [2]	Resorption rate [3]
	g/day	*g/day*	*g/day*
1	0.709	1.249	0.540
2	0.556	0.829	0.273

[1] Determined from increase in weight.
[2] Determined by method of Bauer et al. (3).
[3] Experimental A — Theoretical A.

From these studies it appears that, in the normal beagle dog, the accretion rate does change as a function of age as has been previously reported for other species (3, 7). On the basis of the whole skeleton, calcium deposition rate appears to rise with increasing age after birth to a maximum at about the age of puberty after which it gradually falls until, at maturity, it may be below values obtained at birth. If, however, results are expressed on the basis of unit bone mass, maximum accretion rate values are obtained at the earliest ages studied with deposition decreasing thereafter.

ACKNOWLEDGMENTS

The authors wish to thank Mr. A. R. Beckwith and Mr. R. J. Wallace for their excellent technical assistance.

LITERATURE CITED

1. Jowsey, J., B. A. Lafferty and J. Rabinowitz 1965 Analysis of distribution of Ca^{45} in dog bone by a quantitative autoradiographic method. J. Bone Joint Surg., *47A:* 359.
2. Lee, W. R., J. H. Marshall and H. A. Sissons 1965 Calcium accretion and bone formation in dogs. J. Bone Joint Surg., *47B:* 157.
3. Bauer, G. C. H., A. Carlsson and B. Lindquist 1961 Metabolism and homeostatic function of bone. In: Mineral Metabolism, vol. 1, part B, eds., C. L. Comar and F. Bronner. Academic Press, New York, p. 608.
4. Heaney, R. P., and G. D. Whedon 1958 Radiocalcium studies of bone formation rate in human metabolic bone disease. J. Clin. Endocrinol. Metab., *18:* 246.
5. Aubert, J. P., and G. Milhaud 1960 Méthode de measure des principales voies du métabolisme calcique chez l'homme. Biochim. Biophys. Acta, *39:* 122.
6. Widdowson, E. M., and J. W. T. Dickerson 1964 Chemical composition of the body. In: Mineral Metabolism, vol. 2, part A, eds., C. L. Comar and F. Bronner. Academic Press, New York, p. 2.
7. Green, W. T., H. G. Watts, P. Panagakas, W. Oh, R. L. Wilson and H. H. Banks 1968 Radiocalcium-47 kinetic studies in the dog. Clin. Orthop. Related Res., *61:* 154.
8. Marshall, J. H., R. E. Rowland and J. Jowsey 1959 Microscopic metabolism of calcium in

[4] Bronner, F., R. E. Stacey and S. I. Chang 1969 Calcium metabolism parameters as a function of age and calcium intake. Federation Proc., *28:* 591 (abstract).

bone. V. The paradox of diffuse activity and long-term exchange. Radiat. Res., *10:* 258.
9. Armstrong, W. D., and L. Singer 1965 Composition and constitution of the mineral phase of bone. Clin. Orthop. Related Res., *38:* 179.
10. Wasserman, R. H. 1958 Quantitative studies on skeletal accretion in laboratory and domestic animals. In: Proceedings of the Second International Conference on Peaceful Uses of Atomic Energy, vol. 27, Isotopes in Agriculture. Geneva, p. 132.

Metabolism of Tryptophan in Isolated Perfused Rat Liver [1,2]

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ABSTRACT The fate of tryptophan in the isolated perfused rat liver was investigated. It was shown that the liver removed the L-isomer more rapidly from the perfusate than the D-isomer. The 10 g liver was capable of a removal rate of 35 mg/hour when 42 mg of DL-tryptophan was given initially, followed by infusion at the rate of 42 mg/hour. Carbon-14 from DL-tryptophan-2-^{14}C appeared in CO_2 much more rapidly and to a greater extent within 3 hours than did that from DL-tryptophan-7a-^{14}C. In contrast to whole-animal studies, it was shown that overloading doses of acetoacetate were labeled by DL-tryptophan-5- or 7a-^{14}C. The labeling pattern in acetoacetate was similar to that observed when ^{14}C-fatty acids are the substrate. Acetoacetate overloading suppressed the production of radioactive carbon dioxide from DL-tryptophan-7a-^{14}C.

The central role of the liver in the metabolism of amino acids and in the synthesis and degradation of protein has been demonstrated in part through the use of the isolated perfused rat liver. Comparison of hepatectomized rats and their perfused livers has shown that several amino acids are degraded largely by the liver (1). The liver appears to regulate the level of amino acids circulating in the blood (2–4) and it is responsible not only for the synthesis and degradation of hepatic protein, but for the synthesis and degradation of much of the plasma protein as well (5–8). The isolated perfused liver has been particularly useful in defining the liver's role, since hormonal and other factors controlling amino acid and protein metabolism can be more easily controlled experimentally than in the whole animal and because the activity of extrahepatic tissue is excluded.

Tryptophan is among the amino acids which seem to be degraded largely by the liver (1). This essential amino acid is of particular interest because it is often limiting in the diet of man and animals, and because it is present in mammalian tissues in very small amounts (9). Recently, interest in the fate of tryptophan within animal tissues has been further stimulated by the suggestion that it plays a unique role in the control of protein biosynthesis (10, 11) and in control of gluconeogenesis (12). Therefore, it would be useful to investigate further the metabolism of tryptophan in the liver since this organ is the primary site of gluconeogenesis and is also very active in protein biosynthesis. The isolated perfused rat liver is a particularly convenient system for this study.

METHODS

The liver and blood donors were male albino rats of the Sprague-Dawley strain, fed a commercial laboratory ration [5] and allowed food and water ad libitum. In certain experiments, as indicated, rats fasted for 48 to 72 hours were used as liver donors. The animals were maintained in darkness from 6:00 PM to 6:00 AM in a room with constant temperature and humidity.

Blood donors were retired male breeders weighing approximately 600 g. The animal was placed under anesthesia with diethyl ether, the abdominal cavity was opened, and blood was drawn from the

Received for publication July 17, 1969.
[1] Supported by Grant no. GM 11710 from the National Institutes of Health and by funds provided by the Minnesota Agricultural Experiment Station. Scientific Journal Series No. 6991, Agricultural Experiment Station, University of Minnesota, St. Paul, Minnesota.
[2] Presented in part at the Seventh International Congress of Biochemistry, Tokyo, Japan, August, 1967. Metabolism of tryptophan by perfused rat liver, 1968. Medical Journal of Osaka University, 19: 25 (abstract).
[3] Deceased.
[4] Present address: Nagoya University, School of Medicine, Nagoya, Japan.
[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

abdominal aorta into a syringe containing heparin. The perfusate (40 to 50 ml) consisted of either rat blood or rat plasma which had been diluted one-third to one-half with Ringer's bicarbonate buffer. The perfusate was circulated in the perfusion apparatus for approximately 30 minutes prior to the insertion of the liver, and tryptophan was added 30 to 45 minutes after perfusion began. In some experiments, as indicated, glucose was added to the perfusate in an amount to give an initial concentration of 5 mg/ml. Liver donors ranged in weight from 225 to 325 g, the majority weighing between 275 and 300 g. The livers were removed by a procedure similar to that described by Miller et al. (5). The perfusion apparatus was a slight modification of that described by Green and Miller (7). A mixture of 95% oxygen and 5% carbon dioxide was used for aeration. Carbon dioxide coming from the chamber was trapped in a 1:2 (v:v) mixture of aminoethanol and methoxyethanol. Livers were perfused for a period of 2 to 4 hours and their condition was evaluated on the basis of bile production, rate of perfusate flow and the appearance of the surface.

Carbon-14 was determined in a scintillation counter[6] using the scintillation fluid described previously (13). Plasma and whole blood were dissolved in a 1 N solution of hyamine hydroxide in methanol before the scintillation fluid was added. In some cases, hydrogen peroxide was added with the hyamine hydroxide to decolorize the sample.

The DL-tryptophan-7a-^{14}C and DL-tryptophan-5-^{14}C have been described previously (14, 15) (fig. 1). Other labeled compounds were from commercial sources.[7]

In overloading experiments, plasma was deproteinized with tungstic acid and acetoacetate was isolated by silicic acid chromatography using a modification of the procedure described by Marvel and Rands (16). Two methods were used for the degradation of acetoacetate :1) decarboxylation in an acidic medium containing mercuric sulfate (17), giving acetone as the mercury complex and carbon dioxide whose specific activity was estimated using a vibrating-reed electrometer; and 2) controlled oxidation with potassium permanganate (18) yielding carbon dioxide from carbon-1, formate from carbon-2 and acetate from carbons 3 and 4. Formate was oxidized to carbon dioxide using mercuric sulfate. Acetate was subjected to Schmidt degradation (19).

Tryptophan was assayed chemically by a modification of the procedures of Opienska-Blauth et al. (20) and Fischl (21).

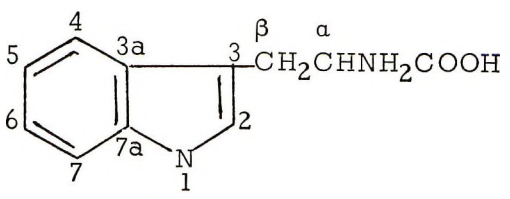

Fig. 1 Numbering system of the tryptophan molecule.

RESULTS

When the disappearance of tryptophan from the perfusate was followed by chemical assay, the pattern shown in figure 2 was obtained. Removal of L-tryptophan was more rapid than that of D-tryptophan, resulting in a lower plasma level of tryptophan after 1 hour. When L-tryptophan was used within approximately 10 minutes

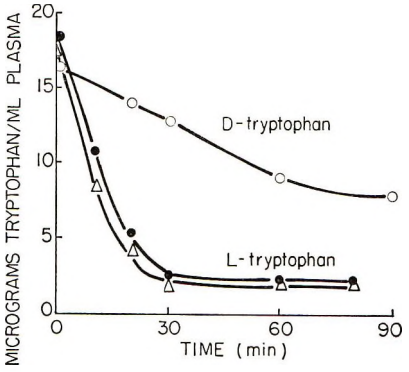

Fig. 2 Rate of uptake of the D- and L-isomers of tryptophan by the isolated rat liver perfused with diluted rat plasma. The liver was perfused for 1 hour to remove endogenous tryptophan from the perfusate. At 1 hour (●———●) and 2.5 hours (△———△), 0.9 mg of L-tryptophan was added. At 4 hours (○———○), 1.1 mg of D-tryptophan was added.

[6] Nuclear-Chicago Model 725, Nuclear-Chicago Corporation, Des Plaines, Ill.

[7] DL-Tryptophan-2-^{14}C was purchased from Tracerlab, Inc., Richmond, Calif.; acetoacetate-3-^{14}C was obtained from Nuclear-Chicago Corporation, Des Plaines, Ill., and glutarate-1,5-^{14}C was from Calbiochem, Los Angeles, Calif.

the plasma tryptophan level fell to about one-half the 15 μg/ml found in the intact animal. The curves in figure 2 were obtained using diluted plasma. As shown in table 1, however, when higher amounts of tryptophan were infused at a constant rate, the removal of DL-tryptophan was more efficient when erythrocytes were present in the perfusion medium. When diluted blood was used, it was necessary to infuse tryptophan at a rate four times higher in order to support a plasma concentration equivalent to that maintained when diluted plasma was used.

The liver removed large amounts of tryptophan from the perfusate; table 1 illustrates the rate of removal when it was infused at high concentrations. This amino acid was not concentrated within the erythrocytes under the conditions used in these experiments. When low concentrations of L-tryptophan (15 μg/ml) were incubated at 37° with diluted whole blood, there was equilibration of the tryptophan between the plasma and red cells within 5 minutes. When larger amounts were used (90 μg/ml), equilibration required a longer time.

The total amount of radioactive carbon dioxide which was produced in 2 to 3 hours by the isolated perfused rat liver depended upon the position of labeling of the tryptophan molecule and to a limited extent upon the amount of tryptophan which was used. As is shown in table 2, there was little or no difference in the amount of radioactive carbon dioxide which was produced when DL-tryptophan-7a-^{14}C was infused at a rate of 21 mg/hour or a rate of 42 mg/hour, since the percentage of ^{14}C in carbon dioxide decreased by a factor of two when the dosage was doubled. More radioactive carbon dioxide was obtained when the ^{14}C was in the carboxyl of the side chain or the 2-position of the indole ring of the tryptophan. Similar yields of $^{14}CO_2$ from C-5 and C-7a were observed (table 3) when tryptophan was given as a single dose at the start of the experiment. Only about 1% of the radioactivity from DL-tryptophan-5-^{14}C was in carbon dioxide compared to 12 to 16% from DL-tryptophan-7a-^{14}C. At very high dosages (150 mg) there was about twice as much radioactivity in carbon dioxide from DL-tryptophan-2-^{14}C as from DL-tryptophan-7a-^{14}C.

The initial rate at which $^{14}CO_2$ was released was also a function of the position of the ^{14}C in the tryptophan given (fig. 3). When the concentration of DL-tryptophan-2-^{14}C in the blood was maintained at 30 μg/ml, production of radioactive carbon

TABLE 1

Effect of rate of infusion of DL-*tryptophan on plasma concentration and liver uptake*

Initial dose	Rate of infusion	Avg plasma concn[1]	Initial rate of uptake [2]
mg	*mg/hr*	*μg/ml*	*mg/hr*
2.0	1.0	30 [3]	2.5
2.0	1.0	4	5
2.0	4.0	37	5
10.3	10.8	110	12
21.0	21.0	410	20
42.0	42.0	584	35

[1] Over the 2-hour period of infusion.
[2] Based on first 30 minutes of perfusion.
[3] Perfusate was diluted plasma; diluted blood was used in other experiments.

TABLE 2

Percentage of ^{14}C appearing in CO_2 during 3 hours as a function of infusion rate and position of the ^{14}C in tryptophan [1]

Substrate	Initial dose	Infusion rate	Percentage of ^{14}C in CO_2
	mg	*mg/hr*	%
DL-Trp-2-^{14}C	2.0	1.0	33.6
DL-Trp-2-^{14}C	2.0	4.2	44.7
DL-Trp-7a-^{14}C	10.3	10.8	6.6
DL-Trp-7a-^{14}C	10.3	10.8	11.8
L-Trp-^{14}COOH	10.3	10.8	21.7
DL-Trp-7a-^{14}C	21.0	21.0	6.5
DL-Trp-7a-^{14}C	21.0	21.0	4.5
DL-Trp-7a-^{14}C	42.0	42.0	3.4
DL-Trp-7a-^{14}C	42.0	42.0	3.4

[1] Perfusate was diluted blood.

TABLE 3

Percentage of ^{14}C from tryptophan appearing in expired CO_2 during 3 hours of liver perfusion

Position of label	Single dose	Percentage of ^{14}C in CO_2
	mg	%
DL-Trp-7a-^{14}C	1.2 [1]	12.6
DL-Trp-7a-^{14}C	1.1	15.0
L-Trp-7a-^{14}C	0.7 [1]	16.3
DL-Trp-5-^{14}C	1.3 [1]	1.2
DL-Trp-7a-^{14}C	150	4.3
DL-Trp-7a-^{14}C	150	7.9
DL-Trp-2-^{14}C	150	16.4

[1] Perfusate was diluted plasma; diluted whole blood was used in other experiments.

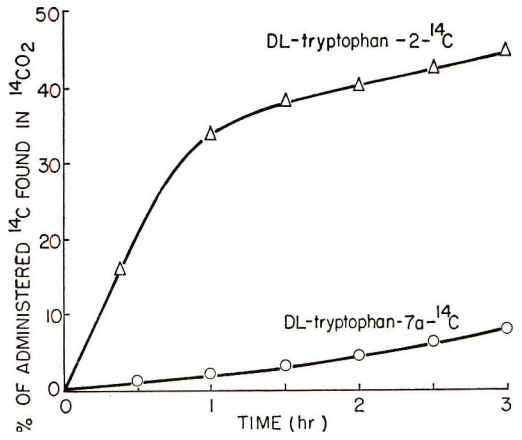

Fig. 3 Rate of release of radioactive carbon dioxide from tryptophan-7a-^{14}C and tryptophan-(ring)2-^{14}C by the isolated perfused rat liver. The initial dose was 2 mg of tryptophan and infusion was at the rate of 4 mg/hour.

dioxide was rapid with 16% of the activity appearing in carbon dioxide after 20 minutes and 45% after 3 hours. Production of radioactive carbon dioxide from DL-tryptophan-7a-^{14}C was slower, with 2% of the activity appearing in carbon dioxide after 1 hour and 8% after 3 hours.

When DL-tryptophan-7a-^{14}C was infused at the rate of 21 mg/hour for 3 hours, about 65% of the carbon-14 was recovered in the perfusate and about 20% in liver. However, only about 20% of the carbon-14 in the perfusate was present as tryptophan. Approximately 6% of the carbon-14 was recovered in carbon dioxide and 2 to 3% in bile. At the infusion rate of 10.8 mg/hour, a larger percentage of the carbon-14 was recovered from carbon dioxide and liver and a somewhat smaller percentage from the perfusate.

Overloading techniques were used to check the usefulness of this procedure in the isolated liver system and to establish the presence of certain intermediates of tryptophan catabolism which have been identified by whole animal studies. The well-documented labeling of overloading doses of glutarate by DL-tryptophan-7a-^{14}C was repeated in the isolated liver. In addition, overloading with 0.5 to 1.0 μmole of sodium acetoacetate led to the isolation of substantial quantities of radioactive acetoacetate from DL-tryptophan-7a-^{14}C or DL-tryptophan-5-^{14}C. When DL-tryptophan-5-^{14}C served as substrate, two radioactive peaks were obtained upon silica gel chromatography of the plasma (fig. 4). The first peak of radioactivity corresponded to the acetoacetate with which the liver

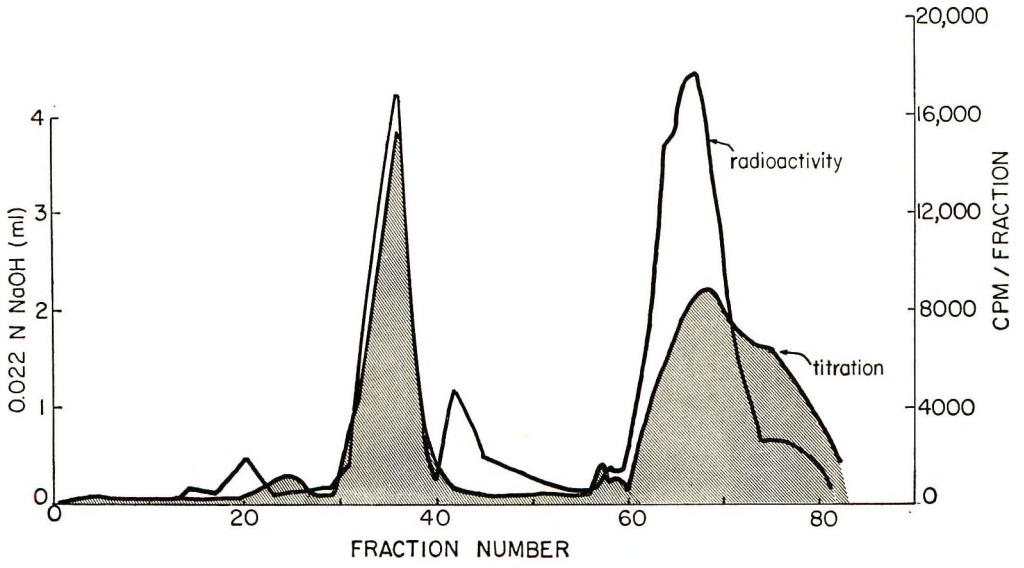

Fig. 4 Silicic acid column elution profile of soluble portion of an isolated rat liver perfused with DL-tryptophan-5-^{14}C and overloaded with acetoacetate. The perfusate was diluted plasma. The first peak corresponds to acetoacetate and the second peak to β-hydroxybutyrate.

was overloaded. The slight displacement of the curve for radioactivity as compared with the titration curve may be an example of an isotope fractionation phenomenon such as has been discussed by Klein (22). The second peak of radioactivity appeared in a region characteristic of β-hydroxybutyrate, although the titration curve showed the presence of other acids in this region. Treatment with β-hydroxybutyrate dehydrogenase indicated that this peak was composed of both the D- and L-isomers, the D-isomer being the major component.

Livers from both fed and fasted rats were overloaded with acetoacetate and the radioactive acetoacetate obtained from DL-tryptophan-7a-^{14}C was degraded. Results of these experiments are shown in table 4.

TABLE 4

Distribution of ^{14}C acetoacetate obtained from DL-*tryptophan-7a-^{14}C using liver perfused with diluted plasma for 2.0 hours* [1]

Treatment	Percentage of ^{14}C in CO_2	Percentage of ^{14}C in acetoacetate	^{14}C Ratio CO/COOH
	%	%	
Fed	2.4	0.4	0.68
Fed	5.6	0.5	0.74
Fed	2.9	0.4	0.82
Fasted	5.6	3.6	0.73
Fasted	3.0	1.6	0.93

[1] 0.82 to 2.95 mg (0.9 to 3.8 μCi) DL-tryptophan-7a-^{14}C was added to perfusate together with 1.0 mmole sodium acetoacetate.

A higher percentage of the carbon-14 was isolated in acetoacetate when livers from fasted rats were used; however, the ratio of radioactivity in the carbonyl carbon to that in the carboxyl carbon appeared not to differ between fed and fasted livers and ranged between 0.7 and 0.9.

When DL-tryptophan-5-^{14}C was perfused and the liver was overloaded with acetoacetate, about 3% of the radioactivity was isolated in acetoacetate (table 5). The ratio of carbon-14 in the methyl to the methylene was approximately 6. The use of glutarate-1,5-^{14}C or acetate-1-^{14}C in similar overloading experiments (table 6) yielded a carbonyl-to-carboxyl carbon-14 ratio similar to that obtained with DL-tryptophan-7a-^{14}C. Glutarate-3-^{14}C yielded a ratio of 10. Perfusion of the liver with very small (1 mg) or very large (1 mmole) amounts of sodium acetoacetate-3-^{14}C resulted in substantial production of radioactive carbon dioxide, and with the high dosage a higher carbonyl-to-carboxyl carbon-14 ratio was obtained than with tryptophan (table 7).

Acetoacetate overloading suppressed the amount of carbon-14 appearing in carbon dioxide from DL-tryptophan-7a-^{14}C (table 4 vs. table 3). Since acetoacetate evidently interfered with production of radioactive carbon dioxide from DL-tryptophan-7a-^{14}C, several brief attempts were made to locate the site of interference. No effect of acetoacetate overloading on tryptophan pyrrolase was noted when the enzyme was measured by in vitro assay following the perfusion (23) or by release of $^{14}CO_2$ from tryptophan-ring 2-^{14}C during perfusion. The combination of 3-hydroxyanthranilate oxygenase and picolinic carboxylase activity, measured by the rate of release of $^{14}CO_2$ from carboxyl-labeled 3-hydroxyanthranilate, was likewise not reduced by the presence of acetoacetate. No effect of acetoacetate on lysine degradation could be detected, suggesting that acetoacetate did not influence the oxidative decarboxylation of α-keto-adipoyl-coenzyme A. The rate of conversion of kynurenine to 3-hydroxyanthranilate was not measured, so it seems

TABLE 5

Distribution of ^{14}C in acetoacetate obtained from DL-*tryptophan-5-^{14}C using liver perfused with diluted plasma* [1]

Treatment	Duration of experiment	Percentage of ^{14}C in CO_2	Percentage of ^{14}C in acetoacetate	^{14}C Ratio CH_3/CH_2
	hr	%	%	
Fed	2.0	< 1	3.1	5.6
Fed [2]	4.0	1.7	2.7	6.8

[1] 1.2 to 1.3 mg DL-tryptophan-5-^{14}C was added to perfusate together with 1.0 mmole sodium acetoacetate.

[2] 500 mg glucose/ml perfusate.

TABLE 6

Distribution of ^{14}C in acetoacetate obtained from glutarate-^{14}C and acetate-^{14}C using liver perfused with diluted plasma for 2 hours

Treatment	Substrate [1]	Percentage of ^{14}C in acetoacetate	^{14}C Ratio CO/COOH
		%	
Fed	Glutarate-1,5-^{14}C	1.6	0.67
Fasted	Glutarate-1,5-^{14}C	2.1	0.45
Fed	Glutarate-3-^{14}C	4.2	10.3
Fed	Acetate-1-^{14}C	6.1	0.87
Fasted	Acetate-1-^{14}C	5.0	0.48

[1] 2 μCi of substrate was added to the perfusate together with 1.0 mmole sodium acetoacetate.

TABLE 7

Utilization of sodium acetoacetate-3-^{14}C by liver perfused for 2 hours with diluted blood

Dosage	Percentage of ^{14}C in CO_2	CO/COOH ^{14}C Ratio in acetoacetate
	%	
< 1 mg	22–24	—
1 mmole	3.8–5.0	37.0

possible that the effect of acetoacetate was on this portion of the pathway (24).

DISCUSSION

The uptake of L-tryptophan by the isolated perfused rat liver appeared to be a rapid process which was directly related to the concentration of L-tryptophan in the perfusate. It was somewhat stereospecific since D-tryptophan was not taken up as rapidly as the L-isomer. However, no evidence was obtained in these experiments for concentrative uptake of tryptophan (compared with plasma levels) by rat erythrocytes or liver. Evidence as to whether or not the isolated rat liver takes up amino acids other than tryptophan against a concentration gradient is limited. Schimassek and Gerok (4) have reported that, when the isolated liver is perfused with an amino acid-free medium, amino acids obtained from protein degradation in the liver are transported to the perfusate. In their studies, after 3 hours of perfusion during which time no amino acids were added, plasma concentrations of threonine, methionine, lysine and histidine were lower than liver concentrations. Valine, isoleucine and leucine were present in higher concentrations in the perfused liver than in whole animals and the concentration of each was approximately the same in the liver and the perfusate. Miller et al. (2) have reported studies in which a mixture of amino acids was added to the perfusate and their removal by the liver was followed. The essential amino acids methionine, phenylalanine, threonine, and lysine appeared to be removed from the perfusate by the liver. However, the levels of valine, isoleucine, leucine and histidine in the perfusate were increased during the 6 hours of perfusion. These investigators did not report the free amino acid levels of the livers, so no comparison of liver and plasma amino acid concentrations can be made. The behavior of valine, leucine and isoleucine in these studies with the isolated liver is probably related to the finding by Miller et al. (1) that the branched-chain amino acids are oxidized to a greater extent by extrahepatic than hepatic tissue. Fisher and Kerly (3) have reported that all amino acids measured were present at a higher concentration in liver than in plasma after 2.5 hours of perfusion without adding amino acids to the perfusate; tryptophan was not measured in their studies. It has been demonstrated that insulin and hydrocortisone (25) and insulin and growth hormone (1) stimulate the uptake of amino acids by the isolated liver. Thus perfusion of rat liver without added hormones may not permit the same type of transport that occurs in the whole animal. With infusion rates of 10 to 42 mg of tryptophan per hour, however, and in the absence of added hormones, liver concentrations of free tryptophan appeared to be nearly equal to plasma concentrations in the present studies. Under these conditions plasma concentrations of tryptophan were much higher than those seen in the whole animal and these high

levels may have masked concentrative uptake.

Kim and Miller (26) have recently reported rates of tryptophan uptake by the isolated rat liver comparable to the initial rates of uptake reported here. When they infused 150 mg of L-tryptophan over a 5-hour period (30 mg/hour) they observed a disappearance rate of 2 mg/g liver per hour in the first 30 minutes. As is shown in table 1, infusion of tryptophan at the rate of 21 mg/hour, after an initial dose of 21 mg, resulted in a disappearance rate of 20 mg/hour per liver in the first 30 minutes. The liver weights in these experiments averaged 10 g; therefore, the disappearance rate in the present studies was 2 mg/g liver per hour, or the same as that reported by Kim and Miller (26). This rapid initial rate of disappearance was not maintained in the present studies, however, as evidenced by increased plasma concentrations of tryptophan, whereas Kim and Miller (26) maintained this rapid uptake throughout the 5 hours of infusion.

The apparent limited capacity of the isolated rat liver to metabolize increasing amounts of tryptophan to carbon dioxide when DL-tryptophan-7a-^{14}C was the tracer, may be related to the lessened ability of the isolated liver to adapt to high levels of tryptophan compared with the whole animal where tryptophan stimulates hydrocortisone secretion. However, some increase in tryptophan pyrrolase activity in isolated liver perfused with high levels of tryptophan has been reported (27), and probably reflects activation of the enzyme (28). In the present studies twice as much $^{14}CO_2$ was obtained from 150 mg of DL-tryptophan-(indole)2-^{14}C as was obtained from 150 mg of DL-tryptophan-7a-^{14}C, which suggests a limited capacity for complete oxidation of the benzene ring of tryptophan. It may be that additional tryptophan is converted to quinolinic acid which is excreted into the perfusate as such, or the benzene ring may be metabolized in greater amounts to nicotinamide mononucleotide and its metabolites, rather than being degraded to carbon dioxide. Kim and Miller (26) have reported $^{14}CO_2$ production in isolated rat liver with infusion of increasing amounts of DL-tryptophan-β-^{14}C. When they infused either 30 mg or 70 mg of tryptophan, about the same amount was coverted to $^{14}CO_2$ in each case, suggesting also a limited capacity for oxidation of the side chain of tryptophan.

Miller et al. (1) obtained about 9% of the radioactivity from DL-tryptophan-α-^{14}C in carbon dioxide during perfusion of rat liver. This is similar to data obtained in this laboratory with 10 mg of DL-tryptophan-α-^{14}C infused during 2 hours of perfusion,[8] although lack of information in the former study (1) as to dosage and length of experiment prohibits direct comparison. A more extensive investigation of the degradation of tryptophan in the isolated perfused rat liver was undertaken by Altman and Gerber (29) who used L-tryptophan-^{3}H and DL-tryptophan-2-^{14}C. They reported only about 10% of the carbon-14 in carbon dioxide after 2 hours of perfusion. This is in contrast to the higher amounts (40% in 2 hours) obtained in the present studies. Although the total dosage was 10 mg in both studies, it was administered differently in that Altman and Gerber gave it at the start of the experiment whereas in the present study only 2 mg was given initially and the remainder was infused at the rate of 4 mg per hour. It does not seem likely, however, that this experimental difference could account for the difference in $^{14}CO_2$ production. The results of Altman and Gerber are similar to those obtained by Miller et al. (1) with DL-tryptophan-α-^{14}C. The suppliers of labeled tryptophan sometimes refer to α-labeled tryptophan as tryptophan-2-^{14}C, so this discrepancy might be explained by a misunderstanding of the position of the label. It was evident that $^{14}CO_2$ production was highest from DL-tryptophan-2-^{14}C and lowest from DL-tryptophan-5-^{14}C, with DL-tryptophan-7a-^{14}C giving intermediate amounts. The data of Kim and Miller (26) suggest that DL-tryptophan-β-^{14}C is intermediate between DL-tryptophan-7a-^{14}C and DL-tryptophan-5-^{14}C in the extent of oxidation to $^{14}CO_2$ in the isolated rat liver.

Tryptophan degradation in the rat involves the conversion of the carbons of the benzene ring to α-ketoadipic acid, fol-

[8] T. Tao and P. Swan. Unpublished results.

lowed by oxidative decarboxylation to glutaryl coenzyme A, further oxidative loss of the free carboxyl carbon (30) to form crotonyl coenzyme A, then L-hydroxybutyryl coenzyme A, acetoacetyl coenzyme A and, finally, acetyl coenzyme A. Thus, the last steps of the degradative pathway for tryptophan are analogous to those for the oxidation of fatty acids. In this sequence of reactions carbon-7a of tryptophan becomes carbon-1 of glutaryl coenzyme A and, hence, the carboxyl carbon of acetoacetyl coenzyme A and acetyl coenzyme A. However, a small portion of glutaryl coenzyme A equilibrates with glutaric acid, resulting in some randomization of the label between carbons 1 and 5 of glutaryl coenzyme A (31) and subsequent loss of the label in carbon-5 as $^{14}CO_2$. Thus, it would be predicted that acetyl coenzyme A arising from tryptophan-7a-^{14}C would be labeled only in the carboxyl carbon, and if the isolated acetoacetic acid were formed entirely from direct condensation of acetyl coenzyme A molecules, it should have a carbonyl-^{14}C/carboxyl-^{14}C ratio of 1. Likewise, tryptophan-5-^{14}C would label acetyl coenzyme A in the methyl carbon and condensation would result in acetoacetic acid with a methyl-^{14}C/methylene-^{14}C ratio of 1. However, the carbonyl-^{14}C/carboxyl-^{14}C ratios for acetoacetic acid formed from tryptophan-7a-^{14}C, glutaric acid-1,5-^{14}C and acetate-1-^{14}C were all lower than 1. Conversely, the methyl-^{14}C/methylene-^{14}C ratios for acetoacetic acid formed from tryptophan-5-^{14}C and the carbonyl-^{14}C/carboxyl-^{14}C ratios for acetoacetic acid formed from glutaric acid-3-^{14}C were both higher than 1. These results are in agreement with those obtained in studies of labeling patterns in acetoacetic acid formed from oxidation of fatty acids (32). It was suggested that there is preferential retention of the methyl terminal 2-carbon unit of fatty acids bound to the thiolase. Hence, when the methyl end is labeled, condensation of this acetyl group with acetyl coenzyme A results in retention of label in the carbons 3 and 4 of acetoacetyl coenzyme A. On the other hand, label in all other carbons of fatty acids is diluted to a greater extent because of free equilibration of these carbons with the cold acetyl coenzyme A pool. Free acetoacetic acid may result from direct cleavage of acetoacetyl coenzyme A (33) or through formation and subsequent cleavage of β-hydroxy-β-methylglutaryl coenzyme A (34). In the latter case, carbons 3 and 4 of acetoacetyl coenzyme A are retained as carbons 3 and 4 of acetoacetic acid, but carbons 1 and 2 of acetoacetyl coenzyme A are exchanged with the acetyl coenzyme A of the cell. This subject has been reviewed by Wakil and Bressler (35).

The production of radioactive carbon dioxide from acetoacetate by the perfused liver in the absence of extrahepatic tissue was of interest. Much of the 1 mmole of acetoacetate used in overloading was not recovered, but it was uncertain whether this represented decarboxylation to give acetone, which was lost by evaporation, or reduction to yield β-hydroxybutyrate. However, when acetoacetate-3-^{14}C was used in small amounts (less than 1 mg), 22 to 24% of the radioactive carbon appeared in carbon dioxide and thus small amounts of the acetoacetate were apparently being oxidized. Perhaps, as has been suggested by McCann (36), acetoacetate was first converted to D-β-hydroxybutyrate (37) which can be converted to the coenzyme A derivative by liver (38).

ACKNOWLEDGMENT

The authors are grateful to R. K. Gholson for supplying the DL-tryptophan-2-^{14}C.

LITERATURE CITED

1. Miller, L. L., W. T. Burke and D. E. Haft 1956 Amino acid metabolism studies with the isolated perfused rat liver. In: Some Aspects of Amino Acid Supplementation, ed., W. H. Cole. Rutgers University Press, New Brunswick, N. J., p. 44.
2. Miller, L. L., W. T. Burke and D. E. Haft 1955 Interrelations in amino acid and carbohydrate metabolism. Studies of the nitrogen sparing action of carbohydrate with the isolated perfused rat liver. Federation Proc., *14:* 707.
3. Fisher, M. M., and M. Kerly 1964 Amino acid metabolism in perfused rat liver. J. Physiol., *174:* 273.
4. Schimassek, H., and W. Gerok 1965 Control of the levels of free amino acids in plasma by the liver. Biochem. Z., *343:* 407.
5. Miller, L. L., C. G. Bly, M. L. Watson and W. F. Bale 1951 The dominant role of the liver in plasma protein synthesis. J. Exp. Med., *94:* 431.
6. Miller, L. L., and W. F. Bale 1954 Synthesis of all plasma protein fractions except

gamma globulins by the liver. J. Exp. Med., *99:* 125.
7. Green, M., and L. L. Miller 1960 Protein catabolism and protein synthesis in perfused livers of normal and alloxan-diabetic rats. J. Biol. Chem., *235:* 3202.
8. Cohen, S., and A. H. Gordon 1958 Catabolism of plasma albumin by the perfused rat liver. Biochem. J., *70:* 544.
9. Wellers, G., J. Chevan and N. Galent 1966 Les "pools" d'acides aminés libres dans les tissus du rat normal. C. R. Soc. Biol., *160:* 739.
10. Fleck, A., J. Shepherd and H. N. Munro 1965 Protein synthesis in rat liver: Influence of amino acids in diet on microsomes and polysomes. Science, *150:* 628.
11. Sidransky, H., D. S. R. Sarma, M. Bongiorno and E. Verney 1968 Effect of dietary tryptophan on hepatic polyribosomes and protein synthesis in fasted mice. J. Biol. Chem., *243:* 1123.
12. Veneziale, C. M., P. Walter, N. Kneer and N. A. Lardy 1967 Influence of L-tryptophan and its metabolites on gluconeogenesis in the isolated, perfused liver. Biochemistry, *6:* 2129.
13. Polan, C. E., W. G. Smith, C. Y. Ng, R. H. Hammerstedt and L. M. Henderson 1967 Metabolism of hydroxylysine by rats. J. Nutr., *91:* 143.
14. Henderson, L. M., D. R. Rao and R. F. Nystrom 1958 DL-Tryptophan-7a-^{14}C. In: Biochemical Preparations, vol. 6, ed., C. S. Vestling. John Wiley & Sons, Inc., New York, p. 90.
15. Mathur, G. P., C. Y. Ng and L. M. Henderson 1964 The synthesis and metabolism of DL-tryptophan-5-^{14}C. J. Biol. Chem., *239:* 2184.
16. Marvel, C. S., and R. D. Rands 1950 Separation of organic acids. J. Amer. Chem. Soc., *72:* 2642.
17. Schepartz, B., and S. Gurin 1949 The intermediary metabolism of phenylalanine labeled with radioactive carbon. J. Biol. Chem., *180:* 663.
18. Weinhouse, S., and R. H. Millington 1949 Ketone body formation from tyrosine. J. Biol. Chem., *181:* 645.
19. Phares, E. F. 1951 Degradation of labeled propionic and acetic acids. Arch. Biochem. Biophys., *33:* 173.
20. Opienska-Blauth, J., M. Charezinski and J. H. Berbec 1963 A new, rapid method of determining tryptophan. Anal. Biochem., *6:* 69.
21. Fischl, J. 1960 Quantitative colorimetric determination of tryptophan. J. Biol. Chem., *235:* 999.
22. Klein, P. D. 1966 The occurrence and significance of isotope fractionation during analytical separations of large molecules. In: Advances in Chromatography, vol. 3, eds., J. G. Giddings and R. A. Keller. Marcel Dekker, Inc., New York, p. 3.
23. Knox, W. E., and V. H. Auerbach 1955 The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem., *214:* 307.
24. Shastri, N. V., S. G. Nayuda and M. C. Nath 1967 Effect of acetoacetate and β-hydroxybutyrate on the biosynthesis of niacin from tryptophan. J. Vitaminol., *13:* 47.
25. Chambers, J. W., R. H. Georg and A. D. Bass 1965 Effect of hydrocortisone and insulin on uptake of α-amino-isobutyric acid by isolated perfused rat liver. Mol. Pharmacol., *1:* 66.
26. Kim, J. H., and L. L. Miller 1969 The functional significance of changes in activity of the enzymes, tryptophan pyrrolase and tyrosine transaminase, after induction in intact rats and in the isolated, perfused rat liver. J. Biol. Chem., *244:* 1410.
27. Price, J. B., and L. S. Dietrich 1957 The induction of tryptophan peroxidase in the isolated perfused liver. J. Biol. Chem., *227:* 633.
28. Knox, W. E. 1966 The regulation of tryptophan pyrrolase activity by tryptophan. In: Advances in Enzyme Regulation, vol. 4, ed., G. Weber. Pergamon Press, New York, p. 287.
29. Altman, K. I., and G. B. Gerber 1967 Catabolism of tryptophan by the isolated, perfused liver and intestine. Nature, *213:* 911.
30. Besrat, A., C. E. Polan and L. M. Henderson 1969 Mammalian metabolism of glutaric acid. J. Biol. Chem., *244:* 1461.
31. Meghal, S. K., H. S. Cheung, R. M. O'Neal and R. E. Koeppe 1966 Metabolism of DL-lysine-2- and -6-^{14}C in rats and dogs. J. Biol. Chem., *241:* 2622.
32. Geyer, R. B., M. Cunningham and J. Pendergast 1950 Acetoacetic acid formation in vitro from odd and even numbered fatty acids. J. Biol. Chem., *185:* 461.
33. Segal, H. L., and G. K. K. Menon 1961 Acetoacetate formation from acetoacetyl coenzyme A in liver mitochondria: Effect of endocrine state and nature of the system. J. Biol. Chem., 236: 2872.
34. Lynen, F., U. Henning, C. Bublitz, B. Sorbo and L. Kroplin-Rueff 1958 Der chemische Mechanismus der Acetessigsäurebildung in der Leber. Biochem. Z., 330: 269.
35. Wakil, S. J., and R. Bressler 1962 Fatty acid metabolism and ketone body formation. Metabolism, *11:* 742.
36. McCann, W. P. 1957 The oxidation of ketone bodies by mitochondria from liver and peripheral tissues. J. Biol. Chem., 226: 15.
37. Azzone, G. F., L. Ernster and E. D. Weinbach 1963 Succinate-linked acetoacetate reduction. I. Endergonic reduction of acetoacetate by succinate in liver mitochondria. J. Biol. Chem., 238: 1825.
38. Lehninger, A. L., and G. D. Greville 1953 The enzymic oxidation of *d*- and *l*-β-hydroxybutyrate. Biochim. Biophys. Acta, *12:* 188.

Evaluation of Dietary Protein Quality in Adult Rats [1]

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ABSTRACT The usefulness of the slope-ratio technique for the evaluation of the nutritional quality of proteins for maintenance rather than growth has been examined using adult rats. Of the four proteins examined, lactalbumin, casein, soy protein, and gluten, the first three produce the responses expected. Gluten, low in lysine, is much more efficiently utilized for maintenance than for growth. Apparently there are mechanisms which permit a relative conservation of body protein when proteins low in lysine (and perhaps other amino acids) are fed at or below maintenance levels. The inadequacies of the slope-ratio method for the evaluation of such proteins are shared by the measurement of biological value, net protein ratio, and net protein utilization. The general applicability of amino acid scores is also questioned since the patterns of amino acids required for growth and maintenance are apparently different. Estimates of the amount of different proteins required for maintenance may be derived from the dose-response regression lines.

It is generally assumed that the nutritive value of proteins is dependent upon the amounts of the essential amino acids they contain relative to the amounts of these amino acids required by the animal consuming them. The classic paper of Block and Mitchell (1) showed that there was a fair correlation between the experimentally determined biological values and the most limiting amino acid when compared to the composition of egg protein. The possibility exists that egg protein might contain some essential amino acids in excess of the requirement and not be an entirely appropriate standard. Thus, the FAO Expert Committee (2) proposed a "provisional pattern" of amino acids based largely upon experimental estimates of the amino acid requirements of man. The second FAO/WHO Expert Group (3) had doubts about this approach and, while suggesting that data were insufficient to distinguish between the biological value of whole milk, human milk or egg protein, recommended that the essential amino acid pattern of egg protein be used as the reference standard.

Although it is recognized that the nutritive value of proteins will vary for different species with different amino acid requirements, rats and chicks, for example, the assumption is made that the nutritive value for monogastric animals is essentially the same. Mitchell (4) has emphasized the similarity of the biological values obtained with rats, dog, swine and man, and attributes the failure to conform of some data obtained with man to experimental difficulties in obtaining accurate estimates. On the other hand, in his monograph Mitchell (5) proposed that amino acid requirements may be broken down into those required to replace endogenous losses, those required for growth, and those required for synthesis of tissues which grow throughout life, such as skin and hair. He then stated: "It seems obvious that the amino acid requirements for these three purposes are different, qualitatively and quantitatively."

If this thesis is correct, it follows that the nutritive value of proteins would be different for growing and adult animals. This is contrary to the usual assumption that the pattern of amino acids required for infants and adults is essentially the same. As Hegsted (6) pointed out, the actual data available upon the amino acid requirements are such that one can easily defend either proposition, i.e., that they are similar or dissimilar. Some of the estimates of amino acid requirements are so

Received for publication May 28, 1969.
[1] Supported in part by Public Health Service Research Grants no. AM-09520 and 5-K6-AM-18455 and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health.

poor that they simply do not provide reliable quantitative values of amino acid needs (7).

There is, in fact, substantial evidence that the pattern of amino acid needs may be quite different for young and adult rats. The early work of Osborne and Mendel (8–10) pointed to a difference in utilization of certain proteins during growth and adult life. Burroughs et al. (11) measured nitrogen balance in adult rats deprived of each essential amino acid for short periods of time. They concluded: "For the replacement of endogenous losses of nitrogen, the adult rat does not need the following dietary amino acids: lysine, leucine, histidine, arginine and phenylalanine." These authors also called attention to the conclusion of Osborne and Mendel (8) that "lysine is not needed to the same degree as tryptophan for maintenance." However, Neuberger and Webster (12) reported the apparently contradictory result that adult rats lost weight rapidly and constantly when deprived of lysine. These authors did suggest that the relative proportions of essential amino acids required for growth and for maintenance might vary considerably.

More recently, Bender (13) measured net protein utilization (NPU) in young animals when each essential amino acid was removed from a complete amino acid mixture. He concluded that proteins devoid of lysine had an NPU of approximately 40%; that proteins completely lacking tryptophan, threonine, histidine, phenylalanine, leucine or isoleucine have NPU values of about 20%; and that only proteins lacking valine or the sulfur amino acids gave the expected value of zero. These results recall the finding of Mitchell and Block (1) that gelatin has a biological value of about 25%. It should be appreciated that these results are quite incongruous since, in theory at least, a protein with an NPU of 40% can meet the protein needs of an animal when fed in an amount 2.5 times the minimal protein need. Similarly, if 5 times the minimal protein need is supplied by proteins with an NPU of 20%, the protein requirement should be met. It is doubtful whether the protein needs can ever be satisfied by feeding more of a protein which is lacking in an essential amino acid. There must be something wrong with either the theory or the method.

Hegsted and Worcester (14) and Hegsted et al. (15) have discussed the errors involved in the bioassay of proteins according to the slope-ratio technique described by Hegsted and Chang (16, 17). For some proteins it was quite clear that the zero intercept of the regression lines relating weight gain, body water or body nitrogen to the amount of protein eaten did not meet the Y-axis at the value obtained with the group fed no protein. This was indicated in the analysis of variance by a significant departure from the blanks.

It should be noted that the measurement of biological value (18), of net protein utilization (19), and of net protein ratio (20) are all based upon measuring the difference in nitrogen retention during a nitrogen-free period and the test protein period. In essence, they constitute a slope-ratio assay based upon two points only, a blank and an experimental point. In all of these tests, it is tacitly assumed that nitrogen retention is linearly related to the amount of test protein fed. If this is not true, the tests are to the same degree invalid. The same assumption is made in the slope-ratio assay, but it offers a means of estimating the degree to which the test departs from the assumption made rather than simply assuming it is true. It also avoids the tendency to (nonevident) variability in the determination of biological value or NPU subsequent to a short low-nitrogen feeding period (21).

The use of amino acid scores to evaluate protein quality also rests on the assumption that the nutritive value should decrease linearly as the level of the essential amino acid falls below the ideal pattern. As has been discussed above, there is much evidence that this assumption is contrary to the facts. The theoretical relationship appears to fail especially when certain essential amino acids are supplied at very low levels or when certain proteins are fed at levels below the maintenance requirement. With young rats it is difficult to investigate this area of the response curve since young animals lose relatively little weight before they become moribund. Thus, we have turned our attention to the response

of adult animals fed protein levels which are near or below maintenance requirements.

This paper reports preliminary attempts to develop a slope-ratio assay for proteins using adult animals and evaluating weight or tissue loss rather than the gain in tissue observed when growing animals are used. Some proteins demonstrate the theoretically expected responses; others do not. Estimates of the requirements of different proteins for maintenance can be made, however.

EXPERIMENTAL

The experimental details are similar to those previously described (14–17) except that adult female rats weighing approximately 200 g were used. These were divided into approximately homogenous groups of five animals each and caged individually. One group of animals was killed on day 1 for carcass analysis. One group (the blanks) received the protein-free diet previously described (14, 15). Predetermined levels of several proteins based on estimates of their probable nutritive value were added to the diets of the other groups as shown in table 1. The feeding period was 31 days. The diets were fed ad libitum; food consumption was measured, and the protein intake calculated. At the end of the experiment the animals were killed and the contents of the stomach and cecums removed. The carcasses were kept frozen in plastic bags for later analysis.

TABLE 1

Kinds and levels of proteins studied

Experiment	Protein source	Level in diet	Actual protein supplied [1] (N × 6.25)
		%	%
1	Lactalbumin	3.0	2.40
	(standard protein)	2.0	1.60
		1.0	0.80
	Wheat gluten	12.0	9.70
		8.0	6.50
		4.0	3.20
2	Lactalbumin	3.0	2.40
	(standard protein)	2.0	1.60
		1.0	0.80
	Wheat gluten	15.0	12.10
		10.0	8.10
		5.0	4.00
	Casein	3.0	2.50
		2.0	1.70
		1.0	0.85
	Soy protein	5.0	2.50
	(cooked)	3.5	1.77
		1.5	0.76

[1] The amount of protein supplied by the conventional proteins was determined by prior Kjeldahl analysis as follows: (in %) lactalbumin, 80.22; gluten, 81.00; casein, 84.94; soy protein, 50.62.

The frozen carcasses were chopped or sliced with a cleaver into relatively small pieces, placed in 400 ml beakers, and dried at 95° to constant weight to determine the total body water. For some large animals, this required 5 to 6 days. The dry carcasses were ground, mixed and a 5 g aliquot heated with 20 ml of 50% sulfuric acid for 3 days at approximately 100°. The hydrolysate was quantitatively transferred to a 500 ml volumetric flask with water and made to volume. Total nitrogen of this solution was determined by the automated Kjeldahl procedure with the AutoAnalyzer [2] and total body nitrogen calculated.

From the body weights and the mean body composition of the animals killed on the first day, the initial body water and body nitrogen of each animal were calculated. These values subtracted from those determined at the end of the experiment yielded the change in body water and body nitrogen for each animal.

RESULTS

Figure 1 shows the changes in body water for each animal in experiment 1 in which lactalbumin and wheat gluten were compared. It is apparent that the change in body water in the animals fed lactalbumin is linearly related to the protein intake, including the animals which received no protein. This is the expected result. The data from the animals fed the wheat gluten diets are also linearly related to the protein intake but this regression line, when extended to the zero intercept, indicates an expected loss of 10 g rather than the determined value of 26 g. Although the data do not define the shape of the gluten curve, it must be curvilinear and approximate the dotted line shown.

[2] Technicon Corporation, Tarrytown, New York.

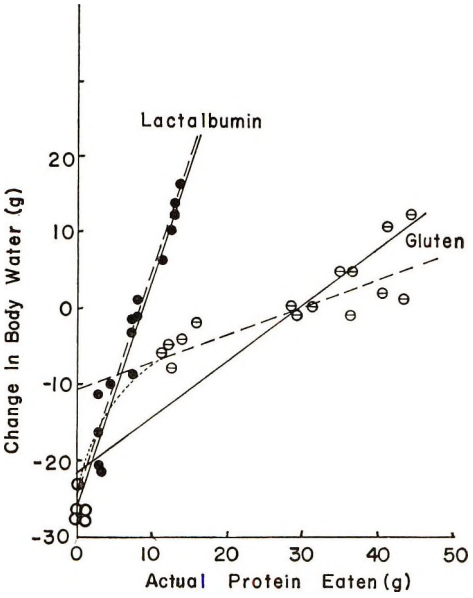

Fig. 1 Regression lines relating the change in body water with protein intake in experiment 1. Solid regression lines calculated with the blanks (zero protein) included. Broken regression lines calculated without the blanks.

Similarly, in experiment 2 (fig. 2) the regression lines obtained with the data from animals fed lactalbumin, casein and soy protein cannot be shown to depart significantly from the expected curves which intercept the Y-axis at approximately the value obtained with the animals fed no protein. On the other hand, the animals fed low levels of gluten do better than expected and the regression line intersects the Y-axis substantially above the blanks.

The figures show only the data on body water. As has long been known, the correlation between body water and body nitrogen is high, usually much above that shown for experiment 2 (table 2). As we have pointed out before (15), the variance of the data for body nitrogen is, in our hands, always greater than that for body water. Body nitrogen is theoretically the best parameter of response to protein utilization. However, we believe that the difficulties in sampling total body carcasses, especially when there is substantial body fat, and the numerous manipulations involved in the nitrogen determinations account for the greater errors in these data. Thus, change in body water is probably a better and certainly a simpler measure of response than body nitrogen.

Table 3 shows the mean NPU values calculated from the body nitrogen determinations and the nitrogen intakes in experiment 1. NPU falls markedly as the level of gluten is increased whereas NPU for albumin is relatively stable. The classical calculation of NPU, however, is relatively unsatisfactory, since there are rather large differences in size of animals in each group relative to the amount of nitrogen retained.

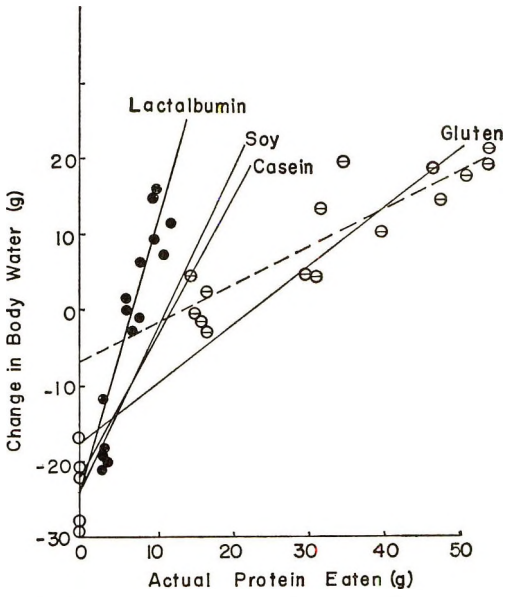

Fig. 2 Regression lines relating the change in body water with protein intake in experiment 2. Solid regression lines calculated with the blanks (zero protein) included. Broken regression line calculated without the blanks. As in experiment 1, the regression lines calculated without the blanks for lactalbumin, casein and soy protein were very close to those calculated with the blanks included. For the sake of simplicity, these lines as well as the data for individual animals receiving casein and soy protein are not shown.

TABLE 2

Correlation coefficients between body water and body nitrogen

Experiment	No.	Correlation coefficients
1	35	0.904
2	65	0.786

TABLE 3

Mean NPU values for experiment 1

Protein	Actual protein supplied	Protein consumed	Protein retained	NPU
	%	g	g	%
Lactalbumin	0.80	3.19	3.02	94.7
	1.60	7.57	8.99	118.8
	2.40	12.68	11.30	89.1
Gluten	3.20	12.13	6.05	46.1
	6.50	32.05	9.21	28.7
	9.70	41.27	8.75	21.2

DISCUSSION

The data shown in figure 1, for example, indicate that at very low levels of intake, approximately 5 g of protein eaten in this particular experiment, the nutritional value of wheat gluten and lactalbumin is approximately the same, i.e., they are about equally effective in preventing modest weight loss. Thus, if one determines the changes in body nitrogen, as in the NPU determination or by nitrogen balance techniques, as a measure of nutritional quality, the nutritional value of gluten will depend upon the levels which the experimenter happens to select. The assay techniques are therefore invalid for this protein.

Similarly, the slope-ratio technique is invalid under these conditions. As has been pointed out before (14), modest departures from the ideal assay in this way are not particularly important when growing animals are used and the proteins are fed at levels which promote substantial rates of gain. The departures from the blanks or intercepts have little effect upon the slope of the dose-response curve. However, when one attempts to apply the same technique to evaluate the nutritional quality of protein for maintenance, as we have here, the technique clearly fails for gluten.

This discrepancy from the expected response is especially important in the evaluation of what Miller and Payne (22) have defined as NPU standard. This is NPU determined at levels near or below maintenance intakes of protein, that range of intake where we conclude that the assay is invalid for certain proteins. Obviously, this accounts for the fact that although Miller and Payne record an NPU of approximately 40 for gluten, Hegsted and Chang (16, 17) found a value of less than 20. Similarly, Hegsted (23) records values for wheat flour and some other cereal proteins considerably less than the NPUs and biological values commonly reported.

The implications of these findings are rather far reaching. Proteins low in lysine appear to be much more efficient in maintenance than they are in the promotion of growth. This has probably been apparent since the work of Osborne and Mendel (8–10). Mitchell and Beadles (24) demonstrated that relatively little lysine was needed for maintenance as compared to the amount required for growth. If it is true for man that the amino acid patterns required for growth and maintenance are quite different, then one cannot apply a single score to proteins in the calculation of protein requirements for different ages. The procedures which have been generally applied (3, 25, 26) would not be entirely defensible. Changes in the lysine requirement with age would also explain the ease with which nitrogen balance can be maintained in adult humans fed cereal diets (27).

Secondly, the ability to estimate protein quality from amino acid scores is questioned. Quite clearly the data are accumulating to show that protein quality as measured by the usual techniques is not lowered proportionately as the level of each essential amino acid falls below the amounts in a "standard protein." Apparently, there are mechanisms which permit a relative conservation of lysine (and perhaps other amino acids) as the

TABLE 4

Estimates of maintenance requirements of different proteins

Experiment	Protein	Estimated maintenance requirement		
		g/day	*% diet* [1]	*% calories*
1	Lactalbumin	0.274	1.77	1.7
	Gluten	0.935	6.58	6.2
2	Lactalbumin	0.226	1.73	1.6
	Gluten	0.419	3.44	3.2
	Casein	0.387	3.39	3.2
	Soy protein (cooked)	0.355	3.06	2.9

[1] The food intake of rats which approximately maintained their original body water ranged from 11.4 to 15.5 g/day, average 13 g/day.

intake becomes low, or there may be mechanisms which permit a conservation of body protein at low intakes of a low-lysine protein which do not occur when a lysine-rich protein is fed.

The rate of depletion is, of course, no measure of essentiality. It may require months to deplete a person of vitamin A compared to days or weeks to deplete the body of thiamine. Nevertheless, if rates of depletion of essential amino acids are markedly different, it is clear that the evaluation of protein quality becomes enormously complicated in animals fed submaintenance or maintenance levels of protein.

Estimates of the maintenance requirements of different proteins are the protein intakes where the regression line intersects a line parallel to the X-axis through the point representing zero change in body water. The values thus estimated for the different proteins tested are given in table 4. Estimates for gluten protein are obtained from the regression lines calculated without the blanks. The estimated maintenance requirements for proteins of high nutritive value are in agreement with the estimate of Osborne and Mendel (28) and approximate the estimated maintenance requirement for man (29).

LITERATURE CITED

1. Block, R. J., and H. H. Mitchell 1946-1947 The correlation of the amino acid composition of proteins with their nutritive value. Nutr. Abstr. Rev., *16:* 249.
2. Food and Agriculture Organization 1957 Protein Requirements. FAO Nutritional Studies no. 16, Report of the FAO Committee, Rome, Italy.
3. World Health Organization 1965 Protein Requirements. Report of a Joint FAO-WHO Expert Group, WHO Technical Report Series no. 301, Geneva, Switzerland.
4. Mitchell, H. H. 1954 The dependence of the biological value of food proteins upon their content of essential amino acids. Wiss. Abh. Deut. Akad. Landwirt., Band V/2, 279.
5. Mitchell, H. H. 1962 The maintenance requirement of protein. In: Comparative Nutrition of Man and Domestic Animals, vol. 1. Academic Press, Inc., New York, pp. 129–191.
6. Hegsted, D. M. 1964 Protein requirements. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison, Academic Press Inc., New York.
7. Hegsted, D. M. 1963 Variation in requirements of nutrients–amino acids. Federation Proc., 22: 1424.
8. Osborne, T. B., and L. B. Mendel 1916 The amino acid minimum for maintenance and growth, as exemplified by further experiments with lysine and tryptophane. J. Biol. Chem., 25: 1.
9. Osborne, T. B., and L. B. Mendel 1916 A quantitative comparison of casein, lactalbumin and edestin for growth or maintenance. J. Biol. Chem., 26: 1.
10. Osborne, T. B., and L. B. Mendel 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., 37: 557.
11. Burroughs, E. W., H. S. Burroughs and H. H. Mitchell 1940 The amino acids required for the complete replacement of endogenous losses in the adult rat. J. Nutr., *19:* 363.
12. Neuberger, A., and T. A. Webster 1945 Lysine requirements of the adult rat. Biochem. J., 39: 200.
13. Bender, A. E. 1961 Determination of the nutritive value of proteins by chemical analysis. In: Progress in Meeting Protein Needs of Infants and Preschool Children. National Academy of Sciences — National Research Council, Pub. 843, Washington, D. C., p. 407.
14. Hegsted, D. M., and J. Worcester 1966 Assessment of protein quality with young rats. Proc. 7th Int. Cong. Nutr., *4:* 1.
15. Hegsted, D. M., R. Neff and J. Worcester 1968 Determination of the relative nutritive value of proteins. Factors affecting precision and validity. J. Agr. Food Chem., *16:* 90.
16. Hegsted, D. M., and Y. Chang 1965 Protein utilization in growing rats. I. Relative

growth index as a bioassay procedure. J. Nutr., *85:* 159.
17. Hegsted, D. M., and Y. Chang 1965 Protein utilization in growing rats at different levels of intake. J. Nutr., *87:* 19.
18. Mitchell, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., *58:* 873.
19. Miller, D. S., and A. E. Bender 1955 The determination of the net utilization of proteins by a shortened method. Brit. J. Nutr., *9:* 382.
20. Bender, A. E., and B. H. Doell 1957 Biological evaluation of proteins: A new concept. Brit. J. Nutr., *11:* 140.
21. Mitchell, H. H. 1948 The biological utilization of proteins and protein requirements. In: Proteins and Amino Acids in Nutrition, ed., M. Sahyun. Reinhold Publishing Corporation, New York, pp. 46–81.
22. Miller, D. S., and P. R. Payne 1961 Problems in the prediction of protein values of diets. The influence of protein concentration. Brit. J. Nutr., *15:* 11.
23. Hegsted, D. M. 1969 Nutritional value of cereal proteins in relation to human needs. In: Protein-Enriched Cereal Foods for World Needs, ed., M. Milner. American Association of Cereal Chemists, Inc., St. Paul, Minn., pp. 38–48.
24. Mitchell, H. H., and J. R. Beadles 1950 Biological values of 6 partially purified proteins for the adult albino rat. J. Nutr., *40:* 25.
25. Hegsted, D. M., A. G. Tsongas, D. M. Abbott and F. J. Stare 1946 Protein requirements of adults. J. Lab. Clin. Med., *31:* 261.
26. Food and Nutrition Board 1964 Recommended Dietary Allowances, ed. 6. National Academy of Sciences — National Research Council, Washington, D. C.
27. Bolourchi, S., C. M. Friedmann and O. Mickelsen 1968 Wheat flour as a source of protein for adult human subjects. Amer. J. Clin. Nutr., *21:* 827.
28. Osborne, T. B., and L. B. Mendel 1915 The comparative nutritive value of certain proteins in growth, and the problem of the protein minimum. J. Biol. Chem., *20:* 351.
29. Hegsted, D. M. 1968 Normal protein requirements in man. In: Nutrition in Renal Disease, ed., G. M. Berlyne. E. & S. Livingstone, Ltd., London, pp. 1–16.

Absorption of Vitamin E by the Rat from a Low Fat Diet

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ABSTRACT To determine whether absorption and utilization of vitamin E are as efficient on a low fat diet as on a diet containing abundant quantities of fat, groups of rats were fed for 8 weeks with low or high fat diets supplemented with graded levels of *d*-α-tocopheryl acetate (0 to 7.61 IU/100 g diet). Erythrocytes from animals fed the low fat diets were less susceptible to hemolysis by dialuric acid than those from animals fed the high fat diets. Hepatic storage of the supplemented vitamin E was not influenced by dietary fat except at the highest dose administered, at which level the high fat diet enhanced storage. Thus, by both criteria, vitamin E is effectively absorbed and utilized whether the animal is fed a low or a high fat diet.

The definition and standard of identity for nonfat dry milk fortified with vitamins A and D (1) has omitted the inclusion of vitamin E even though the recommendations of the National Research Council's Food and Nutrition Board (2) leave little doubt about the importance of this vitamin. In addition to need, however, restoration of essential nutrients requires demonstration of satisfactory utilization from the proposed vehicle.

Prior experimental work with mammals, such as that of Christensen et al. (3, 4), has not answered the question specifically because dietary fat levels were much lower than in nonfat dry milk or because supplements of vitamin E were much higher than the level found in milk. The available clinical data concerning the absorption of vitamin E from low fat diets are not decisive either. Panos et al. (5) observed a prompt response to daily doses of 25 to 50 mg of vitamin E in infants fed a fat-free diet for 40 days, whereas Barness et al. (6) suggested that erratic responses to vitamin E included in low fat formulas for up to 80 days may have resulted from the low intake of total fat and of unsaturated fatty acids.

This experiment was designed to determine whether absorption and utilization of relatively low levels of vitamin E are as efficient on a low fat diet as on a diet containing abundant quantities of fat.

MATERIALS AND METHODS

Diets. Six groups of rats were fed a low fat diet and another six groups received a high fat diet. Graded levels of vitamin E as *d*-α-tocopheryl acetate were added to five of each type of diet. The composition of the diets is summarized in table 1.

A description of the critical dietary ingredients follows.

Nonfat dry milk was made by a high heat spray process.[1] Based on previous work (7), this ingredient contained approximately 0.7% fat, with 4.9% polyunsaturated fatty acids (PUFA) and 29 μg of α-tocopherol per gram fat.

Safflower oil was molecularly distilled [2] to reduce the α-tocopherol content to zero (assayed by thin-layer chromatography (8)). The PUFA content was 76.6% by gas-liquid chromatography (7), and the peroxide value was 7.9 (9).

Coconut oil came from two sources (see footnotes, table 1). The coconut oil used for the first 3 weeks in diets 7 to 12 had a peroxide value of 6.4, an α-tocopherol content of 7.5 μg/g, and 2.0% PUFA. The mixture of coconut oils used in diets 13 to 18 had a peroxide value of 3.4, an α-tocopherol content of 9.7 μg/g, and 2.2% PUFA.

Received for publication August 4, 1969.

[1] Processed by the Dairymen's League Cooperative Association, Inc., Vernon, New York.

[2] Distilled through the courtesy of Mr. Eugene Mularz, Production Department, Distillation Products Industries, Rochester, New York.

TABLE 1

Diet composition

Ingredients	Low fat diets 1 to 6	High fat diets	
		7 to 12	13 to 18
	%	%	%
Nonfat dry milk	54.0	54.0	54.0
B-vitaminized casein [1]	2.0	2.0	2.0
Cornstarch [2]	38.44	5.24	—
Cellulose [3]	5	23.2	25.94
Stripped safflower oil	0.5	0.5	—
Coconut oil	—	15.0 [4]	18.0 [5]
Iron and copper salts [6]	0.06	0.06	0.06
Vitamin E [7]	+	+	+
Vitamins A and D [8]	+	+	+

[1] B-vitamins are premixed with vitamin-test casein to supply the following: (in μg/10 g diet) thiamine HCl, 100; riboflavin, 100; pyridoxine·HCl, 100; vitamin B_{12} (crystalline USP), 0.2; *d*-biotin, 2; folic acid, 20; *d*-calcium pantothenate, 300; nicotinic acid, 500; menadione, 50; and: (in mg/10 g diet) choline chloride, 10; i-inositol, 5; *p*-aminobenzoic acid, 1.

[2] Clinton Corn Processing Company, Clinton, Iowa.

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

[4] Coconut oil was Cochin (Pacific Vegetable).

[5] Coconut oil was mixture of 40% Cochin (Pacific Vegtable) and 60% Cobee (Drew Chemical).

[6] Salt premix: ferric ammonium citrate, 174 g; cupric sulfate (USP) ($\times 5H_2O$), 6 g.

[7] *d*-α-Tocopheryl acetate was dissolved in the stripped safflower oil (diets 1 to 6 and diets 7 to 12) or in the coconut oil (diets 13 to 18) to provide levels of 0, 0.36, 0.76, 1.64, 3.53 or 7.61 IU vitamin E, sequentially in each series, per 100 g finished diet.

[8] Vitamin A palmitate and vitamin D (Viosterol) were added to appropriate oils when diets were mixed to provide 40 and 4 IU, respectively, per gram diet.

The composition of these diets was predicated on several assumptions and estimates. The level of 1.64 IU of vitamin E per 100 g diet corresponds closely to the level supplied by the 90.7 g nonfat solids contained in 1 quart of milk fortified with 1.49 IU vitamin E. This level approximates the maximum seasonal levels of vitamin E observed in homogenized milk (7). The level of 0.5% stripped safflower oil insures adequate nutrition with essential fatty acids (about 39 mg/10 g diet) without elevating the total fat content per 100 g dry diet (0.89%) greatly above that observed per 100 g nonfat dry milk solids (0.73%).

Although the high fat diet should contain approximately 30% fat to provide the same proportion of fat to α-tocopherol as is found in the best seasonal homogenized milk, the more practical level of 15% added fat was chosen initially. For the first 3 weeks of the experiment, the high fat diets (7 to 12) provided 15.89% of total fat and 67 mg PUFA/10 g diet. When hemolysis assays after 2 weeks showed higher hemolysis on the high fat diet than on the low fat diet, the PUFA content of the former was reduced further to 39 mg/10 g diet by eliminating the safflower oil and increasing the coconut oil to 18.0% (diets 13 to 18) to give a total fat content of 18.39%. These diets replaced diets 7 to 12 for the remaining 5 weeks of the experiment.

A high caloric density of fat in the high fat diet could result in a significantly depressed consumption relative to the low fat diet. To prevent this, the high fat diets were diluted with sufficient cellulose to make them essentially isocaloric with the low fat diets, with caloric densities of 3.80 for the low fat diets and 3.81 for the high fat diets.

Animals. Weanling female rats descended from the Sprague-Dawley strain [3] were fed a vitamin E-free diet for 4 days; then 180 rats ranging in weight from 59 to 69 g were divided into 12 groups of 15 rats each and were fed the experimental low and high fat diets for 8 weeks.

All rats were weighed and observed weekly. The average amount of food consumed in a 24-hour period was determined weekly for each group after week 1 of the experiment.

Analyses. The susceptibility of erythrocytes to hemolysis by dialuric acid was determined by the method of Friedman et al. (10). Hemolysis bioassays were

[3] Blue Spruce Farms, Altamount, New York.

done at the end of weeks 2, 4, 6 and 8 on three rats per group. In groups that showed any indication of hemolysis, additional rats were checked. At the end of the experiment, livers were removed from all rats. The livers were frozen and stored at −20° until analyzed for α-tocopherol (8).

RESULTS

Growth and mortality. Growth was normal and comparable in all groups of rats (table 2). Neither the level of dietary fat nor the addition of graded levels of vitamin E had a significant effect. Four scattered deaths occurred during the first 3 weeks of the experiment, one on a low fat diet (group 4) and three on high fat diets (groups 14, 15, 17).

Food consumption. The average daily food consumptions (13 g) were comparable for the groups of rats fed the low and high fat diets during the first 3 weeks of the experiment. After the high fat diet was modified to lower its polyunsaturated fatty acid content at the beginning of week 4, the average daily food consumption per rat was approximately 1 g less on the high (14 g) than on the low fat (15 g) diets.

Hemolysis. Results from the hemolysis bioassay at 8 weeks are summarized in table 2. Rats fed either the low or high fat diet with no added vitamin E showed complete hemolysis (92 to 99%) of red blood cells throughout the experiment. Addition of 0.36 IU vitamin E/100 g diet reduced hemolysis, particularly on the low fat diet; and addition of 0.76 IU/100 g diet eliminated hemolysis on the low fat diet and reduced it to borderline significance on the high fat diet. All higher levels of vitamin E protected the erythrocytes from hemolysis.

Liver analyses. Analyses of livers from various groups (table 2) showed α-tocopherol even in animals with completely hemolyzing red cells. Liver levels increased when the dietary supplement was 1.64 IU/100 g diet or greater. Compared with the low fat diet, the addition of coconut oil significantly increased liver stores of α-tocopherol at all levels of dietary supplementation. Only with the highest supplement, however, was any part of this increase due to the fat per se rather than the additional α-tocopherol contained in the coconut oil.

DISCUSSION

Results of the hemolysis tests after 2 weeks suggested that absorption of vitamin E was less on the high than on the low fat diet. An alternate explanation was that the higher content of polyunsaturated

TABLE 2

Effect of dietary fat and vitamin E on growth, erythrocyte hemolysis and liver tocopherol in female rats

Diets	Group	Vitamin E added [1]	Weight gain [2,3]	Hemolysis [4]	Liver tocopherol [3]	
					Total	Net [5]
		IU/100 g	*g/rat*	*%*	*μg/liver*	
Low fat	1	0	134±4.0 [6]	93±3 (6)	74±11	—
	2	0.36	127±3.7	28±3 (15)	—	—
	3	0.76	126±2.7	1±0 (9)	62±6	0
	4	1.64	134±2.9	1 (3)	118±6	44
	5	3.53	135±3.6	0 (3)	139±9	65
	6	7.61	123±3.7	1 (3)	231±19	157
High fat	13	0	125±3.9	99±1 (6)	121±21	—
	14	0.36	122±4.0	68±6 (14)	—	—
	15	0.76	134±4.3	12±2 (14)	107±16	0
	16	1.64	137±3.7	0 (6)	165±12	44
	17	3.53	129±4.8	3 (3)	176±14	55
	18	7.61	122±4.9	0 (3)	351±23	230

[1] Added as *d*-α-tocopheryl acetate.
[2] Average initial weight was 62 g.
[3] Fifteen rats per group except 14 rats in groups 4, 14, 15 and 17.
[4] Numbers in parentheses indicate animals tested.
[5] Increase in liver tocopherol due to dietary supplement of d-α-tocopheryl acetate.
[6] Mean ± SEM.

fatty acids in the high fat diet increased the requirement for vitamin E, thus obscuring any difference due to level of dietary fat per se. The high fat diets were then adjusted to balance the content of PUFA.

Harris and Embree (11) have estimated that a dietary vitamin E/PUFA ratio of 0.6 mg/gram should be indicative of adequate vitamin E nutriture. With the low fat diet, complete hemolysis was observed in the absence of supplemental vitamin E (group 1), as might be expected with a vitamin E/PUFA ratio of 0.03. Hemolysis diminished to 28% in group 2, with a vitamin E/PUFA ratio of 0.65 and disappeared in group 3 with a ratio of 1.34.

With the high fat diet, hemolysis decreased from 99% to 68% to 12% to 0% in groups 13, 14, 15 and 16, respectively, as the vitamin E/PUFA ratio increased from 0.47 to 1.09 to 1.78 to 3.28, respectively. The apparent increase in hemolysis due to additional dietary fat (but not polyunsaturated fatty acid) and in spite of higher vitamin E/PUFA ratios recalls a previous report by Century and Horwitt (12), who observed that addition of aerated coconut oil to chicken diets containing 2% stripped corn oil aggravated the incidence of encephalomalacia. These workers suggested that provision of additional fat calories as readily metabolizable lauric and myristic acids "freed" more polyunsaturated fatty acids for retention (and peroxidation) in the tissues.

The net hepatic storage of vitamin E from the dietary supplement was not influenced by the level of fat except at the highest supplement of 7.61 IU/100 g diet. At this level, storage was significantly higher with the high fat diet; but significant storage occurred even with the low fat diet.

Although our studies are not long-term, they have spanned the maturation period of the female rat. By both criteria examined (erythrocyte hemolysis and hepatic storage), vitamin E was effectively absorbed and utilized whether the animal was fed a low fat or a high fat diet.

LITERATURE CITED

1. Food and Drug Administration, Department of Health, Education and Welfare 1968 Nonfat dry milk fortified with vitamins A and D; order establishing definition and standard of identity. Federal Register, *33:* 10926.
2. Food and Nutrition Board, National Research Council 1968 Recommended Dietary Allowances, publ. 1694. National Academy of Sciences, Washington, D. C.
3. Christensen, F., and H. Dam 1956 Reproduction capacity of rats in relation to diet. 3. Influence of diets containing no added fat with and without vitamin E and certain artificial antioxidants. Acta Physiol. Scand., *36:* 82.
4. Christensen, F., H. Dam, R. A. Gortner, Jr., and E. Sondergaard 1956 "In vitro" hemolysis of erythrocytes from vitamin E deficient rats and chicks. Acta Physiol. Scand., *35:* 215.
5. Panos, T. C., B. Stinnett, G. Zapata, J. Eminians, B. V. Marasigan and A. G. Beard 1968 Vitamin E and linoleic acid in the feeding of premature infants. Amer. J. Clin. Nutr., *21:* 15.
6. Barness, L. A., F. A. Oski, M. L. Williams, G. Morrow III and S. B. Arnaud 1968 Vitamin E response in infants fed a low fat formula. Amer. J. Clin. Nutr., *21:* 40.
7. Herting, D. C., and E. E. Drury 1969 Vitamin E content of milk, milk products and simulated milks: relevance to infant nutrition. Amer. J. Clin. Nutr., *22:* 147.
8. Herting, D. C., and E. E. Drury 1967 Thin-layer chromatography with precoated alumina sheets. II. Application to tocopherols. J. Chromatog., *30:* 502.
9. Association of Official Agricultural Chemists 1965 Official Methods of Analysis, ed. 10. George Banta Company, Inc., Menasha, Wisconsin, p. 419.
10. Friedman, L., W. Weiss, F. Wherry and O. L. Kline 1958 Bioassay of vitamin E by the dialuric acid hemolysis method. J. Nutr., *65:* 143.
11. Harris, P. L., and N. D. Embree 1963 Quantitative consideration of the effect of polyunsaturated fatty acid content of the diet upon the requirements for vitamin E. Amer. J. Clin. Nutr., *13:* 385.
12. Century, B., and M. K. Horwitt 1959 Effect of fatty acids on chick encephalomalacia. Proc. Soc. Exp. Biol. Med., *102:* 375.

Influence of Feeding Cottonseed Oil to Laying Hens on the Low Density Lipoproteins of Their Eggs [1,2]

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ABSTRACT Low density lipoproteins, isolated from yolks of eggs produced by hens fed a normal diet and from those of hens fed 2.5% cottonseed oil in the diet, were studied to determine the effect of crude cottonseed oil on properties of the yolk lipoproteins. Low density lipoproteins isolated from eggs produced by hens fed crude cottonseed oil contained more saturated fatty acids and less monoenoic fatty acids than did those isolated from normal eggs. Differences in amino acid content of the vitellenins from normal and "cottonseed" eggs were small. When low density lipoproteins were separated into six fractions by ultracentrifugation, most of those from normal eggs were in the top four fractions, but most from the cottonseed oil eggs were in the bottom three fractions. Cottonseed oil low density lipoproteins were on the average larger in size than the normal ones as determined by gel filtration through a Bio-gel A-15m column. The floating fraction of normal low density lipoproteins isolated by ultracentrifugation contained more lipid and the molecules were larger than the soluble low density lipoproteins in the bottom fraction, and the lipoproteins in intermediate fractions were intermediate in lipid content and size of molecule. Cottonseed oil low density lipoproteins were also separated according to molecular size, but the different fractions were similar in lipid content.

Lipids extracted from eggs produced by hens fed crude cottonseed oil contained more stearic acid and less oleic acid than lipids from eggs produced by hens fed a normal ration (1). Evans et al. (2) observed that the low density lipoproteins (LDL) of 6-month old eggs from hens fed crude cottonseed oil migrated almost three times as far as those from fresh eggs when subjected to paper electrophoresis, and that part of the lipovitellin was converted to a lipoprotein that behaved similarly to LDL under the conditions used for electrophoresis. Burley (3) recently reported that LDL isolated from yolks of hens fed methyl sterculate, one of the active components of crude cottonseed oil, were more viscous than those from normal egg yolks.

The purpose of the present investigation was to further study the properties of the low density lipoproteins of eggs from hens fed cottonseed oil to determine if any differences exist between them and normal low density lipoproteins which might explain the increased mobility of the low density lipoproteins of stored eggs from hens fed cottonseed oil.

EXPERIMENTAL

Eighteen Single Comb White Leghorn hens were housed in laying cages; 9 were fed a basal ration [4] and 9 the basal ration to which 2.5% of crude cottonseed oil was added. The basal ration contained 4.1% lipid, and the fatty acids were composed of 17% palmitic, 3% oleic, and 45% linoleic. The fatty acid composition of crude cottonseed oil was 28% palmitic, 2% stearic, 17% oleic, and 51% linoleic.

Low density lipoproteins (LDL) were prepared from 6 freshly layed eggs as described by Evans et al. (4) by a procedure essentially that of Martin et al. (5). Two

Received for publication July 14, 1969.

[1] Michigan Agricultural Experiment Station Journal Article no. 4790. Supported in part by Public Health Service Research Grant AM 10214 from the National Institute of Arthritis and Metabolic Diseases.

[2] Presented in part at the 53rd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 13 to 18, 1969. Federation Proc., *28*: 369 (abstract).

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[4] The percentage composition of the basal ration was: ground corn, 34.5; ground oats, 20.0; wheat bran, 15.0; flour middlings, 10.0; dehydrated alfalfa (15% protein), 3.0; meat scrap (50% protein), 3.0; dried milk, 2.0; menhaden fish meal (60% protein), 2.5; soybean oil meal (44% protein), 2.5; ground oyster shell flour, 5.0; steamed bone meal, 1.5; salt, 0.6; and fish oil (3000 IU vitamin A and 400 IU vitamin D), 0.4.

preparations were made from eggs of hens fed the normal ration and two from eggs of hens fed the ration containing crude cottonseed oil. Hens fed the crude cottonseed oil had been on experimental diet for 19 days when eggs for the first preparation were obtained and for 54 days when those for the second were obtained.

The LDL solution in 1.0 M sodium chloride was dialyzed against distilled water and the resulting solution was centrifuged [5] for 16 hours at 165,000 × *g* and 5°. The contents of each tube were divided into four fractions, the solid floating fraction, the top half of the liquid, the bottom half of the liquid, and the solid pellet of residue (fig. 1). The floating solid fraction was dispersed in distilled water, and the residue was suspended in distilled water. Each fraction was then centrifuged as above; the contents of tube F (floating fraction) were divided into the solid floating part and the liquid portion; the contents of tube R (residues) were divided into the supernatant and residue; and the contents of the other tubes were divided into the top and bottom halves. The liquid portion of tube F and the top portion of tube A were combined as were the bottom portion of tube A and the top one of tube B, and the bottom portion of tube B and the supernatant of tube R (fig. 2). These fractions were again centrifuged and further fractionated as shown in figure 2 to give six fractions.

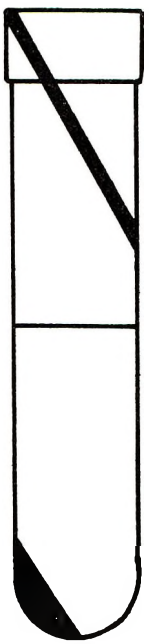

Fig. 1 Fractionation of egg yolk LDL solution in water by centrifugation in the Beckman-Spinco Model L ultracentrifuge using the No. 50 Ti rotor at 50,000 rpm for 16 hours at 5°. The top layer is solidified LDL and was designated fraction F. The bottom fraction is a pellet of sedimented precipitate. The solution was divided into two equal portions.

Bio-gel A-15m and Bio-gel A-50m were used to fractionate the LDL by gel-filtration through agarose bead columns. Columns were 2.5 cm in diameter and approximately 90 cm long, and they were jacketed and operated at a temperature of 1 to 3° with upward flow to minimize excessive packing of the columns. Columns were eluted with 1.0 M sodium chloride solution at a flow rate of 20 ml per hour. The eluate was collected in 5.0 ml fractions by use of a drop-counter attachment and a refrigerated fraction collector. A constant flow rate was maintained with an LKB peristaltic pump. The proteins were located either by measurement of the ultraviolet absorption of each fraction at 280 mμ in a spectrophotometer [6] or by monitoring the effluent as it passed through a cell in a spectrophotometer [7] connected to a recorder.[8]

An aliquot of the dialyzed unfractionated LDL and portions of each fraction obtained

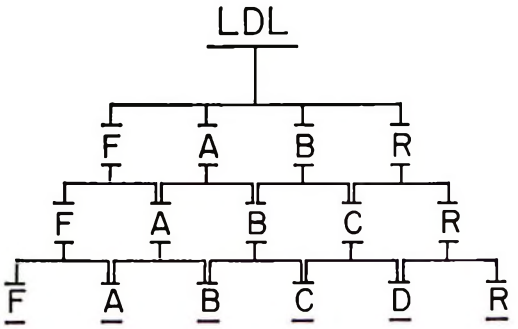

Fig. 2 Scheme for the fractionating of LDL. LDL in water solution was centrifuged and separated as described in the text.

[5] Beckman-Spinco Model L ultracentrifuge using the 50 Ti rotor, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
[6] Beckman Model DU, Beckman Instruments, Inc., Fullerton, Calif.
[7] Beckman Model DB, Beckman Instruments, Inc., Fullerton, Calif.
[8] Sargent Model SRL, Sargent-Welch Scientific Company, Chicago, Ill.

by ultracentrifugation were lyophilized and weighed to determine the weight of LDL in the fraction or aliquot of LDL. The dry LDL was extracted with chloroform–methanol (1:1, v/v) to remove lipid, and the extracted lipid was weighed after removal of the solvent. The lipids were saponified, the fatty acids converted to the methyl esters, and the fatty acid methyl esters separated by gas-liquid chromatography as described previously (1).

The protein residues were hydrolyzed with constant boiling hydrochloric acid in evacuated sealed tubes at 110° for 24 hours (4). Amino acid contents of the hydrolysates were determined with an amino acid analyzer [9] using a procedure based on the Piez and Morris (6) modification of the Spackman, Stein and Moore (7) method.

Solutions of normal and "cottonseed-oil" LDL were treated with one of the following before extraction with ether (4) or extracted without treatment: 8 M urea, 6 M guanidine hydrochloride, 0.0026 M sodium dodecyl sulfate, 2% sodium deoxycholate, 0.13 M β-mercaptoethanol and 8 M urea.

Solutions of normal and cottonseed-oil LDL were digested with phospholipase C or with phospholipase D as described previously (4).

RESULTS

LDL isolated from eggs of hens fed the basal ration contained 87.0% lipid and 10.7% protein (determined on the residue by micro-Kjeldahl procedure), values quite similar to those of 85.8 and 11.7%, respectively, for LDL isolated from eggs of hens fed the ration containing crude cottonseed oil.

The amino acid contents of the proteins are given in table 1. Any differences observed in amino acid contents of vitellenin (LDL apoprotein) from normal eggs and from eggs of hens fed cottonseed oil were small and were probably caused by experimental error.

The fatty acids obtained from eggs of hens fed cottonseed oil contained a much higher percentage of the saturated fatty acids (stearic and palmitic) and a lower percentage of the monounsaturated fatty acids (palmitoleic and oleic) than did those from eggs of hens fed the basal ration (table 2). The differences were largely in stearic and oleic acids.

TABLE 1

Amino acid content of vitellenin from eggs of hens fed a basal diet and a diet containing crude cottonseed oil

Amino acid	Normal	Cottonseed oil
	g/16 g N	
Aspartic acid	11.3	10.9
Threonine	6.6	6.7
Serine	6.6	7.0
Glutamic acid	15.7	16.3
Proline	3.3	4.0
Glycine	3.0	3.0
Alanine	5.6	5.4
Valine	6.5	6.6
Methionine	1.9	2.1
Isoleucine	6.9	6.7
Leucine	10.1	10.4
Tyrosine	5.6	5.3
Phenylalanine	5.5	5.5
Lysine	9.2	9.2
Histidine	1.6	1.8
Arginine	7.4	7.0

TABLE 2

Fatty acid content of low density lipoprotein lipids from eggs of hens fed a basal diet and a diet containing crude cottonseed oil

Fatty acid	Normal	Cottonseed oil
	% of total	
Myristic, C_{14}	0.6	0.7
Palmitic, C_{16}	24.8	27.9
Palmitoleic, $C_{16}{}^{=}$	3.2	1.8
Stearic, C_{18}	8.6	21.8
Oleic, $C_{18}{}^{=}$	47.8	32.3
Linoleic, $C_{18}{}^{=}{}^{=}$	15.0	15.6

Distributions of LDL from normal eggs produced by hens fed the basal ration and from eggs of hens fed crude cottonseed oil into the six fractions are presented in table 3. Most of the LDL from normal eggs was in the top four fractions while most of the LDL from "cottonseed" eggs was in the bottom three fractions. About 54% of the LDL from cottonseed eggs was in the residue. The floating solid fraction F contained the most lipid and the residue or fraction D the least, with the lipid content of the others in between, and each of the fractions of cottonseed LDL contained more lipid than the corresponding fraction of normal LDL. The relative molecular weights of the LDL in each fraction were larger for the cottonseed than for the normal and this was particularly true in the bottom fractions (fig. 3). Molecules of

[9] Technicon Amino Acid Analyzer, Technicon Corporation, Ardsley, New York.

TABLE 3

Weight, percentage lipid, and percentage protein of fractions obtained by ultracentrifugation of LDL from normal and "cottonseed" eggs

Fraction	Weight		Lipid		Protein	
	Normal	Cottonseed	Normal	Cottonseed	Normal	Cottonseed
	g	*g*	%	%	%	%
F	3.09	0.14	89.1	92.2	8.4	6.5
A	5.90	0.83	87.8	89.1	9.2	9.7
B	5.79	1.83	85.9	87.7	10.0	10.5
C	4.04	2.80	84.6	86.5	14.6	10.8
D	1.54	5.05	83.5	85.5	13.9	11.1
R	0.75	12.55	84.1	84.7	12.2	11.8

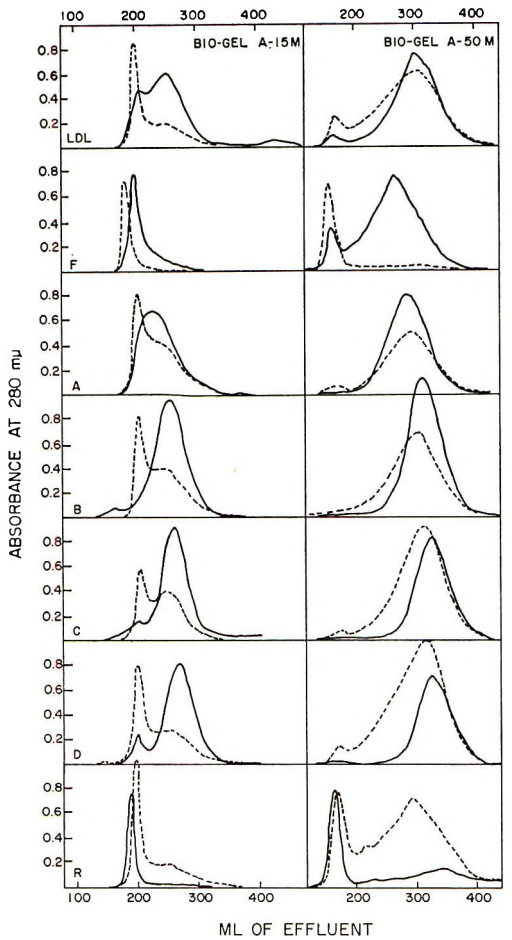

Fig. 3 Gel-filtration through a Bio-gel A-15m column or a Bio-gel A-50m column of normal LDL (————) and of "cottonseed-oil" LDL (– – – – – –) or their fractions in 1.0 M sodium chloride solutions.

LDL fraction F of normal eggs were much larger in size than LDL fraction D molecules and the others were in between, but there was not very much difference in size of those from cottonseed eggs except that molecules of the very small amount of fraction F appeared to be much larger than molecules of the other fractions when estimated by gel-filtration through Bio-gel A-50m.

The various fractions of LDL from normal eggs contained percentages of amino acids (table 1) and fatty acids (table 2) similar to those of the unfractionated LDL, as did those from cottonseed eggs.

TABLE 4

Extraction of lipids by ether from LDL of normal and "cottonseed-oil" eggs after various treatments

Treatment	Normal	"Cottonseed oil"
	% of total lipid extracted	
None	13	12
Urea	7	9
Guanidine·HCl	6	7
Sodium dodecyl sulfate	26	27
Sodium deoxycholate	4	5
β-Mercaptoethanol	61	64
β-Mercaptoethanol + urea	52	58

TABLE 5

Extraction of lipids by ether from LDL of normal and "cottonseed-oil" eggs after digestion with phospholipases

Enzyme	Normal	"Cottonseed oil"
	% of total lipid extracted	
None	10	7
Phospholipase C	51	45
Phospholipase D	10	8

Comparisons of amounts of lipid extracted from normal and cottonseed-oil LDL by ether, either before any treatment of the LDL or after treatment or enzymic digestion, showed no differences in the percentage of lipid extracted (table 4 and 5) and presumably in the lipoprotein structure (4).

Gel-filtration of either normal or cottonseed-oil LDL through a Bio-gel A-15m column separated the LDL essentially into two fractions (fig. 3). Cottonseed-oil LDL appeared to contain a larger proportion of the higher molecular weight fraction than did normal LDL. Gel-filtration through a Bio-gel A-50m column showed a somewhat different distribution of LDL than did gel-filtration through the Bio-gel A-15m column, but the cottonseed-oil LDL here also appeared to have a larger proportion of higher molecular weight LDL than did the normal.

The effluents from gel-filtration separations of normal and cottonseed-oil LDL on a column of Bio-gel A-15m were each separated into five fractions as shown in figure 4. Four different separations of both lipoproteins were made, and the fractions for each protein were pooled. Weight of the fraction and the lipid content of the fraction are given in table 6. Cottonseed-oil LDL contained more of the larger molecular weight fraction, but normal LDL contained more of the smaller fractions.

DISCUSSION

Most of the lipid of the chicken egg yolk occurs as part of the low density lipoprotein (8) and the lipid is composed of 72 to 76% of sterols and glycerol esters (4, 5, 8). Evans et al. (4) presented data supporting the view that the low density lipoprotein is a sphere of lipid, mostly neutral lipids, surrounded by a layer of protein and phospholipid. Most of the neutral lipids of egg yolk occur in the low density lipoproteins (8). Eggs layed by hens fed crude cottonseed oil as part of their ration contained a higher proportion of saturated fatty acids and a lower proportion of monoenoic fatty acids than did eggs produced by normal hens (1). The increase of saturated fatty acids occurred only in the neutral lipids and not in the phospholipids. For these reasons one would expect that the low density lipoproteins would be those most influenced by feeding cottonseed oil.

The similarity of amino acid contents of vitellenin from normal eggs and of that from eggs of hens fed cottonseed oil (table 1) indicates that differences in properties of the low density lipoproteins are not caused by differences in the protein constituent. Because lipid from low density

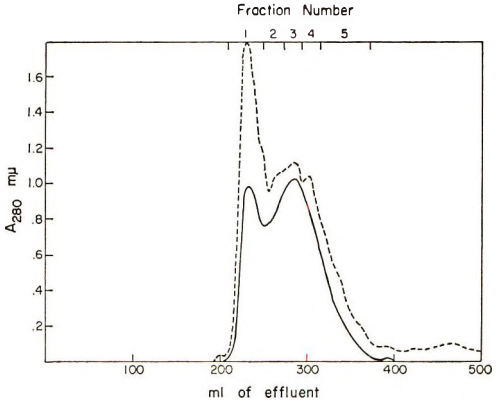

Fig. 4 Gel-filtration through a Bio-gel A-15m column (2.5 × 90 cm) of normal LDL (————) and of "cottonseed-oil" LDL (– – – – – –) in 1.0 M sodium chloride solutions.

TABLE 6

Weight and lipid content of fractions of normal and "cottonseed-oil" LDL obtained by gel-filtration through Bio-gel A-15 m

Fraction	Weight		Lipid	
	Normal	"Cottonseed oil"	Normal	"Cottonseed oil"
	mg	*mg*	%	%
1	119.4	180.0	84.3	85.6
2	171.7	137.4	83.8	83.8
3	118.9	75.2	82.0	82.4
4	83.4	77.0	83.3	82.6
5	98.8	55.8	80.4	82.2

lipoproteins isolated from eggs of hens fed cottonseed oil contained more than twice as much stearic acid as that from normal egg low density lipoproteins (table 2), the differences appear to be in the lipid constituent.

Low density lipoproteins from hens fed cottonseed oil were heavier than normal low density lipoproteins because they mostly sedimented in water whereas the normal LDL mostly floated, as shown by the ultracentrifugation studies (table 3). The differences in density must have been caused in some manner by the increased amount of stearic acid and decreased amount of oleic acid in the lipids.

It is not known why LDL molecules from eggs of hens fed crude cottonseed oil were on the average larger (fig. 3) than those from normal eggs. Attempts have not yet been made to break up any association of molecules that might have occurred. It is possible that the larger molecules are composed of smaller subunits and that the subunits of the stearic acid-rich lipoprotein from eggs of cottonseed oil-fed hens associate more readily than do the oleic acid-rich lipoproteins from normal eggs.

Whether all of the differences between normal LDL and cottonseed-oil LDL, such as in molecular size, lipid content, density, and electrophoretic mobility, are caused by the increased saturation of the lipids in the LDL resulting from increased stearic acid and decreased oleic acid in cottonseed-oil eggs is not known, but most of the differences are probably the immediate result of the changed properties of the LDL lipids.

ACKNOWLEDGMENTS

The authors thank Mr. S. Hulkonen for taking care of the eggs and Miss Susan Jo O'Hopp for some of the lipid extraction and enzyme digestion studies.

LITERATURE CITED

1. Evans, R. J., J. A. Davidson and S. L. Bandemer 1961 Fatty acid and lipid distribution in egg yolks from hens fed cottonseed oil or *Sterculia foetida* seeds. J. Nutr., *73:* 282.
2. Evans, R. J., S. L. Bandemer, J. A. Davidson and D. H. Bauer 1959 Protein distribution in fresh and stored shell eggs from hens fed crude cottonseed oil. J. Agr. Food Chem., *7:* 47.
3. Burley, R. W. 1968 Lipid-protein interactions in abnormal egg yolk: a comparison of the major lipoprotein fractions from normal eggs and from the eggs of hens fed methyl sterculate. Can. J. Biochem., *46:* 851.
4. Evans, R. J., S. L. Bandemer, J. A. Davidson, K. Heinlein and S. B. Vaghefi 1968 Binding of lipid to protein in the low-density lipoprotein from the hen's egg. Biochim. Biophys. Acta, *164:* 566.
5. Martin, W. G., N. H. Tattrie and W. H. Cook 1963 Lipid extraction and distribution studies of egg yolk lipoproteins. Can. J. Biochem. Physiol., *41:* 657.
6. Piez, K. A., and L. Morris 1960 A modified procedure for the automatic analysis of amino acids. Anal. Biochem., *1:* 187.
7. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., *30:* 1190.
8. Evans, R. J., and S. L. Bandemer 1961 Lipide distribution in egg yolk lipoprotein complexes. Poultry Sci., *40:* 597.

Effect of a Copper-Molybdenum Compound Upon Copper Metabolism in the Rat [1,2]

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ABSTRACT X-ray diffraction powder analysis was used to identify a copper-molybdenum (Cu-Mo) compound prepared under ordinary laboratory conditions as a synthetic form of the rare natural mineral, lindgrenite. Weanling rats were used in experiments designed to study the metabolic availability of copper from the Cu-Mo compound. In either normal or copper-depleted rats fed the Cu-Mo compound as their only copper supplement, serum ceruloplasmin oxidase activity (CPA) was significantly lower than in rats fed isocupric levels of copper sulfate. When fed with copper sulfate, a quantity of molybdenum (as sodium molybdate) equal to that in synthetic lindgrenite had no effect upon CPA. In normal but not in copper-depleted rats, liver and kidney copper concentrations were reduced when the Cu-Mo compound was fed instead of copper sulfate, either without or with sodium molybdate. Hemoglobin concentrations were repleted more slowly following copper depletion in the lindgrenite-supplemented rats than in the copper sulfate-treated rats. These results suggest that copper in the form of the Cu-Mo compound, synthetic lindgrenite, is metabolically less available than copper from the sulfate salt.

The reciprocal antagonism between copper and molybdenum, that copper can alleviate molybdenum toxicity and that molybdenum can precipitate copper deficiency, is well known (1–6). Previous explanations concerning the mechanism of this metabolic interaction often have adequately explained the effect of one metal upon the other but have not accounted for the reverse phenomenon. It has been reported that a poorly defined copper-molybdenum (Cu-Mo) compound is formed in vitro (7, 8), and that this compound might represent a possible mechanism for the nutritional interaction between copper and molybdenum (9). The purposes of the investigations to be reported in this paper were to characterize the Cu-Mo compound and to study the metabolic availability to the rat of copper from such a compound.

MATERIALS AND METHODS

Characterization of the Cu-Mo compound. The Cu-Mo compound was prepared as a precipitate by mixing aqueous solutions of a cupric salt (chloride, nitrate, or sulfate) and sodium molybdate. Reagent grade chemicals and distilled, deionized water were used for all preparations. The suspended precipitate was centrifuged for 5 minutes at 1000 × *g*; the supernatant solution was discarded; and the precipitate was resuspended in water. This washing procedure was repeated 4 times. The precipitate from the final wash was collected on filter paper and dried in an oven at approximately 80°. The dried powder was used for the various analyses.

The chemical homology of the Cu-Mo compound was determined by preparing the compound from solutions of copper and molybdenum which varied in concentration from 0.05 M to 1.00 M. These samples were analyzed for copper and molybdenum content. Copper was determined colorimetrically by the method of Parks et al. (10), as modified by Matrone et al. (11) except that wet ashing was not necessary. Molybdenum was determined by atomic absorption spectrophotometry.[3]

Complete elemental analyses were performed by a commercial laboratory[4] in

Received for publication August 15, 1969.

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

[2] Some of these data were presented at the 53rd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey 1969 Federation Proc., 28: 300 (abstract).

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

[4] Coors Spectro-Chemical Laboratory, Division of Coors Porcelain Company, Golden, Colorado.

order to determine whether or not any ionic species other than copper and molybdenum had been incorporated into the Cu-Mo compound. Subsequently, X-ray diffraction powder analysis was performed on various Cu-Mo samples.

Metabolic availability studies. Weanling male rats [5] were used in this phase of the investigation. The rats were housed in individual stainless steel wire cages where diet and distilled water were provided ad libitum. In experiment 1, 48 rats were randomly divided into four treatment groups of 12 rats each. The basal diet (< 2 ppm Cu) for this experiment is shown in table 1. The treatments added to the basal diet are presented in table 2. Group 1, which received no copper supplement, served as the negative control; group 2, which received copper sulfate, was the positive control; group 3 received the Cu-Mo compound; group 4, which received copper sulfate and sodium molybdate, was a control for group 3. The supplements provided 6 ppm copper (groups 2, 3, and 4) and 6 ppm molybdenum (groups 3 and 4). The rats were continued on this regimen for 6 weeks, during which time body weights were recorded weekly and hemoglobin concentrations, determined as cyanmethemoglobin, at weeks 2 and 5. At the end of the 6-week period, the rats were killed and serum samples collected. Livers, kidneys, and spleens were also removed and kept frozen until analyzed. The serum was assayed for ceruloplasmin oxidase activity (CPA) by the method of Houchin (12), as modified by Rice (13). The tissues were analyzed for copper concentrations by atomic absorption spectrophotometry [6] following wet ashing in nitric and perchloric acids.

TABLE 1

Composition of basal diet (experiment 1)

Ingredients	Amount
	%
Vitamin-free casein [1]	20.0
Glucose [2]	62.2
Alphacel [1]	5.0
Corn oil [3]	5.0
L-Cystine [1]	0.3
Mineral mix [4]	5.0
Vitamin mix [5]	2.5

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Cerelose, Corn Products Refining Company, New York, New York.
[3] Mazola Oil, Corn Products Company, Englewood Cliffs, New Jersey.
[4] Mineral mix contained: (g/kg diet) $Ca(H_2PO_4)_2 \cdot H_2O$, 16.4; $CaCO_3$, 11.5; KH_2PO_4, 7.6; $MgSO_4 \cdot 7H_2O$, 5.1; $Na_2HPO_4 \cdot 7H_2O$, 1.2; NaCl, 1.0; $MnSO_4 \cdot H_2O$, 0.19; $FeC_6H_5O_7 \cdot 5H_2O$, 0.18; ZnO, 0.014; KI, 0.00023.
[5] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 2

Treatments added to basal diets

Group	Basal diet additive
1	none
2	15 mg $CuSO_4$/kg
3	18 mg Cu-Mo/kg
4	15 mg $CuSO_4$/kg + 16 mg $Na_2MoSO_4 \cdot 2H_2O$/kg

Since it has been reported (4) that the copper status of the rat influences the copper-molybdenum-sulfate interaction, experiment 2 was designed to study this effect. The 48 weanling rats [7] used in this study were copper-depleted for 2 weeks by feeding a diet containing the following: (in percent) whole-milk powder,[8] 97.76; vitamin-mix,[9] 2.20; $MnSO_4 \cdot H_2O$, 0.02; and $FeC_6H_5O_7 \cdot 5H_2O$, 0.02. At the end of the 2-week depletion period, the same treatments as used in experiment 1 were added to the whole-milk powder diet, which contained approximately 1.3 ppm Cu. Hemoglobin concentrations were determined at the end of the depletion phase, and at weeks 3 and 6 of the 6-week treatment phase. Weekly body weights were recorded. At the end of experiment 2, the rats were killed; serum samples were collected; and livers, kidneys, and spleens were excised. Copper and CPA determinations were made as before.

The data were statistically treated using the *t* test (14). Statements of significance indicate $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of the Cu-Mo compound. It was determined that the Cu-Mo compound was chemically homologous by varying the molar concentrations and the molar ratios of the solutions used to prepare the compound. Copper analysis of 51 such

[5] Sprague-Dawley, Inc., Madison, Wisconsin.
[6] See footnote 3.
[7] See footnote 5.
[8] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[9] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 3

Compositional analysis of the Cu-Mo compound [1]

Cupric salt	Composition [2]			
	Mo	Cu	Total H_2O	O
	%	%	%	%
$CuSO_4$	36.37	34.76	0.97	27.90
$Cu(NO_3)_2 \cdot 2H_2O$	34.72	34.30	1.23	29.75
$CuCl_2 \cdot 2H_2O$	34.53	34.01	0.99	30.47
Average	35.21	34.36	1.06	29.37

[1] All samples were prepared with $Na_2MoO_4 \cdot 2H_2O$ and the indicated cupric salt.
[2] Analyses for sodium and the respective cupric anions gave negligible results.

samples indicated a copper content of 33.4%. The molybdenum content of 29 samples was 32.3%. Results of the complete analyses of Cu-Mo compounds prepared from various salts are shown in table 3. The Cu-Mo compound was chemically homologous regardless of the salt. In addition, none of the other ionic species in solution were incorporated into the compound.

Preliminary X-ray diffraction powder analysis of 13 samples of the Cu-Mo compound, again, indicated chemical homology and gave a pattern closely resembling the rare natural mineral, lindgrenite, $2CuMoO_4 \cdot Cu(OH)_2$. Subsequently, a sample of natural lindgrenite was obtained and X-ray diffraction powder patterns from the natural mineral were compared with patterns from the synthetic Cu-Mo compound. The calculated data from the major peaks are presented in table 4. From the X-ray diffraction powder data, the Cu-Mo compound was positively identified as a synthetic form of lindgrenite.

Metabolic availability studies. In experiment 1 there were no significant differences among the treatment groups with respect to growth rates and hemoglobin concentrations. The effects of treatment upon CPA and tissue copper levels in experiment 1 are summarized in table 5. The rats which received synthetic lindgrenite, group 3, showed a significant depression in CPA compared with the other groups which received copper supplements (groups 2 and 4). The lack of a significant difference between group 2 and group 4 would seem to suggest that the effect observed in group 3 was a function of the lindgrenite compound and not simply an influence of molybdenum. The fact that the lindgrenite-treated rats showed greater CPA than rats which received no supplemental copper

TABLE 4

X-ray diffraction powder data for lindgrenite and the Cu-Mo compound

Miller indices			Lindgrenite		Cu-Mo compound
h	k	l	Published [1]	Observed [2]	
			d(*A*)	*d*(*A*)	*d*(*A*)
0	2	0	7.01	6.98	7.09
1	2	0	4.34	4.34	4.35
1	0	$\bar{1}$	4.15	4.16	4.17
0	4	0	3.50	3.51	3.51
1	4	0	2.96	2.96	2.96
2	0	0	2.76	2.77	2.77
0	0	2	2.67	2.67	2.68
1	4	1	2.50	2.51	2.51
2	2	$\bar{1}$	2.46	2.46	2.46
1	2	$\bar{2}$	2.40	2.40	2.41
1	5	1	2.21	2.21	2.13
2	0	$\bar{2}$	2.08	2.08	2.08
1	7	0	1.88	1.89	1.88

[1] Values from Calvert and Barnes (15).
[2] Values from comparative studies with natural lindgrenite.

TABLE 5

Effect of dietary additives upon serum ceruloplasmin oxidase activity and tissue copper concentration [1]

Group [2]	Ceruloplasmin activity	Tissue		
		Liver	Kidney	Spleen
	IU	*ppm on dry weight basis*		
1	1.6 ± 0.91 a [3]	6.5 ± 0.30 a	11.4 ± 0.85 a	1.5 ± 0.92 a
2	30.4 ± 2.62 b	11.9 ± 0.48 b	31.2 ± 3.60 b	5.4 ± 2.06 b
3	25.7 ± 1.08 c	10.7 ± 0.31 c	24.4 ± 1.54 c	7.2 ± 3.69 b
4	29.4 ± 1.37 b	12.3 ± 0.56 b	30.3 ± 1.29 b	6.1 ± 1.76 b

[1] Each value represents the mean of 12 observations with the standard deviation.
[2] Group 1, no copper; group 2, 6 ppm copper as copper sulfate; group 3, 6 ppm copper and 6 ppm molybdenum as the Cu-Mo compound; group 4, 6 ppm copper as copper sulfate and 6 ppm molybdenum as sodium molybdate.
[3] Values in the same column not followed by the same letter are significantly different ($P < 0.05$).

TABLE 6

Effect of a 2-week depletion and 6-week repletion of copper upon hemoglobin concentration [1]

Group [2]	Weeks of repletion [3]		
	0	3	6
	g/100 ml		
1	9.9 ± 1.25 a [4]	10.2 ± 0.68 a	9.3 ± 0.89 a
2	9.9 ± 0.75 a	13.3 ± 0.78 b	14.0 ± 0.58 b
3	9.8 ± 1.23 a	12.1 ± 0.67 c	13.0 ± 0.50 c
4	9.6 ± 0.87 a	13.4 ± 0.57 b	14.1 ± 0.74 b

[1] Each value represents the mean of 12 observations with the standard deviation.
[2] Group 1, no copper; group 2, 6 ppm copper as copper sulfate; group 3, 6 ppm copper and 6 ppm molybdenum as the Cu-Mo compound; group 4, 6 ppm copper as copper sulfate and 6 ppm molybdenum as sodium molybdate.
[3] Zero-time was at the end of the depletion period (beginning of treatment).
[4] Values in the same column not followed by the same letter are significantly different ($P < 0.05$).

(group 1) suggests that a portion of the lindgrenite copper was available for ceruloplasmin synthesis, although it was not as available as copper from the sulfate salt. In addition to having depressed CPA, group 3 rats had significantly reduced liver and kidney copper concentrations compared with the other 2 groups which received copper supplements. This latter observation might suggest that a part of the normal tissue stores of copper had been depleted for metabolic use. Again, the lack of significant differences between group 2 and group 4 in liver and kidney copper concentrations suggests that the group 3 effect was dependent upon the lindgrenite molecule and not merely a molybdenum effect. The copper concentration in the spleens appeared to be slightly higher in the lindgrenite-treated rats but this difference was not significant.

In experiment 2 in which the rats were copper depleted prior to being placed upon treatment, there was, again, no effect of treatment upon growth rate. However, there was a treatment effect upon hemoglobin concentration in experiment 2 as shown in table 6. The zero-time (end of the depletion period) hemoglobin concentrations were below normal which indicated that the depletion phase had been effective in reducing copper nutrition. Following the addition of copper to the diet, hemoglobin levels began to increase. However, the group 3 rats which received lindgrenite did not replete their hemoglobin as rapidly as did the rats which received copper sulfate, either without or with sodium molybdate (i.e., groups 2 and 4, respectively).

The effects of the various treatments upon CPA and tissue copper concentration in the copper-depleted experiment are summarized in table 7. As in experiment 1 the group 3 rats (those which received synthetic lindgrenite as their copper supplement) had significantly reduced CPA. One question to be asked at this point is: Why were the CPAs so much greater in experiment 2 than in experiment 1? The increased CPA amounted to approximately

TABLE 7

Effect of dietary additives following copper depletion upon serum ceruloplasmin oxidase activity and tissue copper concentration [1]

Group [2]	Ceruloplasmin activity	Tissue		
		Liver	Kidney	Spleen
	IU	*ppm on a dry weight basis*		
1	1.7 ± 1.21 a [3]	4.4 ± 0.65 a	8.8 ± 1.08 a	2.3 ± 0.74 a
2	47.2 ± 4.78 b	9.1 ± 1.64 b	32.5 ± 6.14 b	5.8 ± 1.09 b
3	35.1 ± 5.22 c	8.6 ± 0.82 b	29.8 ± 3.57 b	5.9 ± 1.28 b
4	48.8 ± 5.02 b	9.2 ± 1.32 b	29.5 ± 5.06 b	4.5 ± 0.77 c

[1] Each value represents the mean of 12 observations with the standard deviation.
[2] Group 1, no copper; group 2, 6 ppm copper as copper sulfate; group 3, 6 ppm copper and 6 ppm molybdenum as the Cu-Mo compound; group 4, 6 ppm copper as copper sulfate and 6 ppm molybdenum as sodium molybdate.
[3] Values in the same column not followed by the same letter are significantly different ($P < 0.05$).

60% for the groups 2 and 4 rats, but only about 37% for the group 3 rats. One possible explanation for the increased CPA in experiment 2 is that the rats contracted a minor respiratory ailment at about the midpoint of the study. This ailment could account for the greater activity since stress conditions are known to increase CPA (16, 17). Even so, neither the total CPA nor the amount of the CPA increase was as great in the group 3 rats as in the other two copper-treated groups.

Tissue copper concentrations resulting from the copper-depleted experiment are somewhat different from the results of experiment 1. Contrary to the data obtained in that experiment, the copper-depleted study resulted in no significant differences in liver and kidney copper concentrations among rats which received supplemental copper, regardless of the form. The suggested explanation for the reduced tissue copper levels in the lindgrenite-treated rats in experiment 1, was that the normal stores had been depleted for metabolic function. A similar observation would not necessarily be expected in experiment 2, in which copper depletion occurred prior to treatment. In fact, the lack of significant differences in liver copper concentrations between the lindgrenite-treated rats and the other copper-supplemented rats in experiment 2 seems to indicate that lindgrenite copper was readily absorbed and taken up by the tissues. The splenic copper concentration in the group 4 rats, those which received copper sulfate and sodium molybdate, was significantly lower than in the other copper-supplemented rats. An explanation for this observation is not apparent.

To summarize the data concerning the metabolic availability experiments, it appears that dietary copper in the form of the Cu-Mo compound, synthetic lindgrenite, was not as readily available for metabolic use, particularly ceruloplasmin oxidase activity, as was copper from the sulfate salt. This is evidenced by the observations, in both experiments reported, that serum CPA was reduced in rats fed synthetic lindgrenite as their copper supplement, compared with rats fed copper sulfate. The presence of sodium molybdate at a level of 6 ppm molybdenum in a copper-adequate diet appeared to have no effect upon copper metabolism in the rat, regardless of whether or not the rats had been depleted of their copper stores. Thus, it appears that the effects reported herein were a function of the complex formed between copper and molybdenum rather than simply dependent upon the presence of molybdenum in the diet.

The formation of synthetic lindgrenite in vivo is certainly an attractive hypothesis to explain the metabolic interaction between copper and molybdenum. The basic assumption upon which this hypothesis rests is that copper and molybdenum bound in the form of lindgrenite are metabolically inactive. Although a portion of the lindgrenite copper is metabolically available, the data presented herein and those reported earlier (8, 9) suggest that lindgrenite copper is not as readily available for metabolic function as is copper from the sulfate salt. This hypothesis concerning the copper-molybdenum interaction is adequate to explain the reciprocal antagonistic nature of the 2 metals: that molybdenum can initiate copper deficiency, and that copper can alleviate molybdenum toxicity. Such adequacy has been the major deficiency of earlier explanations.

Whether or not lindgrenite can be formed in vivo cannot be ascertained from the present data. The answer to this question must await the development of a suitable and sensitive assay for lindgrenite in biological material.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Norman Witt and Russell Honea (University of Colorado, Boulder) for preliminary X-ray diffraction powder analysis on the Cu-Mo compound; Arthur Gude III and Richard Van Loenen (U. S. Geological Survey, Denver) for the comparative X-ray diffraction powder analysis and computer analysis of the data; Dr. Ralph Cannon (U. S. Geological Survey, Denver) for the loan of the Chuquicamatan lindgrenite sample for the comparative studies; Gerhard Isaac for assistance in statistical

evaluation; and Billy James for assistance in the care of the animals.

LITERATURE CITED

1. Ferguson, W. S., A. L. Lewis and S. J. Watson 1943 The teart pastures of Somerset. I. The cause and cure of teartness. J. Agr. Sci., *33:* 44.
2. Dick, A. T., and L. B. Bull 1945 Some preliminary observations on the effect of molybdenum on copper metabolism in herbivorous animals. Aust. Vet. J., *21:* 70.
3. Miller, R. F., and R. W. Engel 1960 Interrelations of copper, molybdenum and sulfate sulfur in nutrition. Federation Proc., *19:* 666.
4. Gray, L. F., and L. J. Daniel 1964 Effect of the copper status of the rat on the copper-molybdenum-sulfate interaction. J. Nutr., *84:* 31.
5. Gaballah S. S., L. G. Abood, A. Kapsalis and D. Sturdivant 1965 Ceruloplasmin: in vivo inhibition in the rabbit. Proc. Soc. Exp. Biol. Med., *119:* 625.
6. Arthur, D. 1965 Interrelationships of molybdenum and copper in the diet of the guinea pig. J. Nutr., *87:* 69.
7. Neilands, J. B., F. M. Strong and C. A. Elvehjem 1948 Molybdenum in the nutrition of the rat. J. Biol. Chem., *172:* 431.
8. Dowdy, R. P., and G. Matrone 1968 Copper-molybdenum interaction in sheep and chicks. J. Nutr., *95:* 191.
9. Dowdy, R. P., and G. Matrone 1968 A copper-molybdenum complex: its effects and movement in the piglet and sheep. J. Nutr., *95:* 197.
10. Parks, R. Q., S. L. Hood, C. Hurwitz and G. H. Ellis 1943 Quantitative chemical microdetermination of twelve elements in plant tissue. A systematic procedure. Ind. Eng. Chem. (Anal. ed.), *15:* 527.
11. Matrone, G., W. J. Peterson, H. M. Baxley and C. D. Grinnels 1947 Copper and iron in the blood serum of dairy calves. J. Dairy Sci., *30:* 121.
12. Houchin, O. B. 1958 A rapid colorimetric method for the quantitative determination of copper oxidase activity (ceruloplasmin). Clin. Chem., *4:* 519.
13. Rice, E. W. 1962 Standardization of ceruloplasmin activity in terms of international enzyme units. Anal. Biochem., *3:* 452.
14. Snedecor, G. W., and W. G. Cochran 1967 Statistical Methods, ed. 6. Iowa State University Press, Ames, Iowa, p. 91.
15. Calvert, L. D., and W. H. Barnes 1957 The structure of lindgrenite. Can. Mineralogist, *6:* 31.
16. Starcher, B., and C. H. Hill 1965 Hormonal induction of ceruloplasmin in chicken serum. Comp. Biochem. Physiol., *15:* 429.
17. Evans, G. W., D. R. Myron and R. E. Wiederanders 1969 Effect of protein synthesis inhibitors on plasma ceruloplasmin in the rat. Amer. J. Physiol., *216:* 340.

Effect of Protein and Riboflavin on Plasma Amino Acids and Hepatic Riboflavin-coenzymes in the Rat [1]

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ABSTRACT To study the effect of protein quality and riboflavin on the concentrations of free amino acids in plasma and riboflavin-coenzymes in liver, weanling rats were fed diets containing casein, gluten or gluten plus lysine as sources of protein, supplemented with two levels of riboflavin. When compared with data obtained for animals fed 18% casein, the feeding of 18% gluten with an oral administration of 100 μg riboflavin resulted in a depression of plasma lysine, which was markedly greater than that of other essential amino acids, and in a significant increase in threonine. When the gluten diet was supplemented with 10 μg riboflavin, an interaction between riboflavin and protein resulted in a less pronounced change in the concentration of plasma lysine with no apparent change in threonine. A reduction in the essential to nonessential (EN) amino acid ratio in plasma of animals fed gluten was attributed to a decrease of most essential amino acids and to an increase in serine and glycine. Addition of lysine to the gluten diet did not significantly alter the EN ratio. Values for flavin adenine dinucleotide and total riboflavin in liver were significantly less in animals fed gluten than in animals fed casein. Comparison of data obtained for animals fed 18% casein with those of animals fed 18% gluten plus lysine showed that lysine supplementation had no significant effect on riboflavin fractions or total riboflavin of liver. The value observed for free riboflavin plus flavin mononucleotide in liver of animals fed gluten with 10 μg riboflavin was greater than that of animals fed casein or gluten plus lysine. The interaction between dietary riboflavin and protein was significant at the 5% level. The data indicated that although plasma amino acids may have value in evaluating protein status, other factors may be involved.

Specific variations in the concentrations of amino acids in plasma concomitant with changes in amino acid composition of dietary protein are well established. Less precise information is available to show simultaneous effects of marginal levels of other dietary nutrients on plasma amino acids. Since ariboflavinosis is the deficiency disease most often observed among populations who subsist almost entirely on cereal grain deficient in lysine (1), this study was undertaken to provide more factual information on variations in the concentrations of plasma amino acids which result from supplementation of cereal protein with lysine and riboflavin. The relation of changes in plasma amino acids to the concentrations of riboflavin-containing coenzymes in liver tissue was also investigated.

METHODS

Male rats of the Sprague-Dawley strain weighing approximately 60 g were fed riboflavin-deficient diets which provided 18% casein, 18% gluten or 18% gluten supplemented with 1.2% L-lysine hydrochloride. Except for protein the diets were similar in every respect (table 1). The lysine supplement replaced an equivalent amount of sucrose. Six days per week, one-half the animals assigned to each diet received orally 10 or 100 μg riboflavin in aqueous solution. The animals were caged individually and fed ad libitum during the 8-week experimental period.

The method of Tallan et al. (2) was used to measure amino acids in picric acid extracts of plasma from fasted rats. Equal volumes of plasma from four rats assigned to each dietary treatment were pooled, with the exception of the group fed gluten for which equal volumes of plasma from eight

Received for publication June 27, 1969.

[1] Scientific series paper no. 1451, published with the approval of the Director of the Colorado Agricultural Experiment Station.

TABLE 1

Composition of rations

Ingredient	Casein [1]	Gluten [2]	Gluten [2] + lysine
	%	%	%
Protein	18.0	18.0	18.0
L-Lysine·HCl	—	—	1.2
Mineral mixture [3]	4.0	4.0	4.0
Solka Floc [4]	4.0	4.0	4.0
Cottonseed oil [5]	10.0	10.0	10.0
Sucrose	64.0	64.0	62.8
Vitamins [6]	+	+	+

[1] Vitamin free, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[3] United States Pharmacopoeia XIV, 1950.
[4] Brown Company, San Francisco, Calif.
[5] Wesson Oil, Wesson Sales Company, Fullerton, Calif.
[6] Provided in mg per kg of ration: thiamine·HCl, 5.0; pyridoxine·HCl, 2.5; niacin, 10.0; calcium pantothenate, 20.0; biotin, 0.1; folic acid, 2.0; *p*-aminobenzoic acid, 100.0; inositol, 100.0; vitamin B_{12}, 0.02; menadione, 0.50; choline chloride, 1300; and zinc as zinc carbonate, 12. Each rat received weekly 1000 IU vitamin A acetate, 100 IU calciferol and 0.8 mg *dl*-α-tocopheryl acetate.

rats per dietary treatment were pooled. An amino acid analyzer [2] was used to determine the concentration of amino acids. The fluorometric method developed by Bessey et al. (3) was used to measure the concentrations of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in liver tissue. The concentrations of riboflavin-containing coenzymes in liver were calculated as riboflavin. The data were treated statistically by analysis of variance (4).

RESULTS

The mean weight gain of rats fed the diet providing 18% casein supplemented with 10 or 100 μg riboflavin for 8 weeks was 147 ± 5 and 259 ± 5 g, respectively. Supplementation of 18% gluten with 1.2% L-lysine·HCl increased the weight gain of rats fed 10 or 100 μg riboflavin from 42 ± 3 to 129 ± 4 g and from 49 ± 3 to 223 ± 3 g, respectively. Although the level of dietary riboflavin had no significant effect on the concentration of essential amino acids in plasma within dietary treatments, the effect of type of protein on plasma amino acids was related to the level of dietary riboflavin (table 2). When the higher level of riboflavin was fed, the amino acid in plasma most affected by the feeding of gluten was lysine which decreased approximately 65% ($P < 0.01$). A less significant reduction in the concentrations of valine, methionine, leucine and isoleucine ($P < 0.05$) was accompanied by an increase in the concentration of threonine

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

TABLE 2

Effect of protein and riboflavin on free amino acids in plasma

Amino acid	10 μg riboflavin			100 μg riboflavin		
	18% casein	18% gluten	18% gluten + 1.2% lysine	18% casein	18% gluten	18% gluten + 1.2% lysine
	μmoles/100 ml			*μmoles/100 ml*		
Threonine	47.0 ± 3.8 [1]	47.4 ± 3.2	20.8 ± 4.4	38.3 ± 1.3	52.3 ± 2.8	15.1 ± 2.0
Valine	13.0 ± 3.9	9.0 ± 3.1	12.3 ± 2.7	16.9 ± 1.7	9.8 ± 0.9	12.6 ± 1.0
Methionine	4.2 ± 0.4	2.0 ± 0.7	3.3 ± 0.8	4.8 ± 0.8	2.6 ± 0.5	4.7 ± 0.2
Isoleucine	9.0 ± 0.7	5.4 ± 1.2	7.8 ± 2.0	8.2 ± 0.6	5.4 ± 0.6	7.4 ± 0.5
Leucine	12.8 ± 1.2	8.1 ± 1.9	10.4 ± 2.4	11.9 ± 1.1	8.1 ± 0.8	11.4 ± 1.0
Tyrosine	6.3 ± 0.7	3.4 ± 1.2	6.8 ± 0.9	9.0 ± 1.3	4.3 ± 1.0	10.2 ± 0.3
Phenylalanine	5.3 ± 0.5	3.9 ± 1.4	5.0 ± 0.6	5.8 ± 0.8	4.7 ± 0.5	6.6 ± 0.6
Lysine	29.9 ± 2.0	22.0 ± 4.8	34.3 ± 4.3	37.4 ± 2.4	13.2 ± 2.3	40.1 ± 3.0
Histidine	6.9 ± 0.7	6.2 ± 0.7	3.8 ± 1.4	5.8 ± 0.5	6.2 ± 0.7	5.8 ± 1.0
Arginine	10.0 ± 0.5	11.8 ± 1.4	6.9 ± 2.7	12.8 ± 1.1	11.7 ± 1.6	13.4 ± 2.5
Aspartic acid	2.4 ± 0.2	2.4 ± 0.8	2.8 ± 0.9	1.2 ± 0.2	3.4 ± 0.2	2.6 ± 0.3
Serine	36.7 ± 2.9	64.8 ± 6.8	43.9 ± 5.5	36.7 ± 1.4	76.0 ± 6.8	42.6 ± 1.3
Glutamine	58.9 ± 5.3	48.1 ± 5.9	61.5 ± 11.9	58.1 ± 6.4	45.0 ± 15.7	65.5 ± 2.9
Proline	32.9 ± 4.7	28.8 ± 3.6	45.6 ± 6.6	27.8 ± 2.7	16.0 ± 5.6	26.5 ± 2.2
Glutamic acid	17.6 ± 1.8	18.8 ± 3.9	17.2 ± 3.8	17.0 ± 1.0	21.5 ± 1.3	20.6 ± 0.7
Citrulline	4.8 ± 1.7	9.0 ± 3.5	4.6 ± 2.1	8.6 ± 0.4	10.4 ± 0.9	9.6 ± 0.1
Glycine	28.4 ± 3.3	45.6 ± 3.1	33.7 ± 5.5	27.6 ± 3.7	44.9 ± 2.5	38.9 ± 3.6
Alanine	49.1 ± 3.7	37.4 ± 4.5	55.3 ± 7.1	50.3 ± 5.5	39.1 ± 2.2	54.2 ± 4.1
Ornithine	6.0 ± 0.4	7.7 ± 1.1	6.5 ± 0.6	7.0 ± 0.9	7.3 ± 0.5	7.5 ± 1.2

[1] Mean of four samples ± SEM.

which was highly significant ($P < 0.01$). The addition of lysine to gluten significantly increased the concentrations of isoleucine ($P < 0.05$), methionine and lysine ($P < 0.01$) in plasma of animals fed 100 μg riboflavin. The concentrations of amino acids in plasma of animals fed gluten plus lysine did not differ significantly from those of animals fed casein, except for threonine which was lower when the riboflavin supplement was 10 ($P < 0.05$) or 100 μg ($P < 0.01$). This lower level of threonine observed in animals fed the supplemental lysine was the only significant change in essential amino acids in plasma of animals fed 10 μg riboflavin. The higher level of plasma lysine observed in animals fed gluten with 10 μg riboflavin was caused by a significant interaction between protein and riboflavin.

Tyrosine in plasma of animals fed gluten with the higher level of riboflavin was lower than that of animals fed casein ($P < 0.05$) or gluten plus lysine ($P < 0.01$). Proline and citrulline were the only nonessential plasma amino acids affected significantly by the higher level of riboflavin intake which reduced proline ($P < 0.01$) and increased citrulline ($P < 0.05$). Plasma from animals fed gluten contained higher levels of serine and glycine ($P < 0.01$) and lower levels of alanine and proline ($P < 0.01$) than that of other animals.

The essential to nonessential (EN) amino acid ratios are shown in table 3. The EN ratio, which was affected by protein only, was higher for animals fed casein than for corresponding animals fed gluten or gluten plus lysine. Analysis of variance showed that changes in concentration of serine and glycine had the greatest effect in lowering the EN ratios of animals fed gluten. Supplementation of the gluten diet with lysine lowered serine and glycine and increased proline and alanine. Expressed in micromoles per 100 ml plasma, the total essential and nonessential amino acids for all dietary treatments ranged from 381 to 395.

Mean values for the concentrations of total riboflavin, FAD and FMN plus free riboflavin in liver are given in table 4.

TABLE 3

Total amounts and molar ratios of essential and nonessential amino acids in plasma of rats

Diet	Riboflavin	Total amino acids		
		Essential	Nonessential	EN Ratio [1]
	μg	μmoles/100 ml	μmoles/100 ml	
18% Casein	10	144.4	236.8	0.61
	100	150.9	234.3	0.64
18% Gluten	10	119.2	262.6	0.45
	100	118.3	263.6	0.45
18% Gluten+lysine	10	111.4	271.1	0.41
	100	127.3	268.0	0.47
Residual error variance		622.2	1,451.0	0.0053

[1] Molar ratio of essential-to-nonessential amino acids.

TABLE 4

Effect of protein and riboflavin on riboflavin-containing coenzymes in liver of rats

Protein	Riboflavin [1]	Weight gain	Riboflavin fractions in liver (moist wt basis)		
			Total	FMN [2]	FAD
	μg	g	μg/g	μg/g	μg/g
18% Casein	10	147 ± 5 [3]	12.96 ± 0.56 [3]	1.53 ± 0.21	11.43 ± 0.71
	100	259 ± 5	25.83 ± 1.17	4.54 ± 0.43	21.29 ± 1.08
18% Gluten	10	42 ± 2	12.45 ± 0.55	2.41 ± 0.23	10.04 ± 0.64
	100	49 ± 3	18.71 ± 0.78	3.97 ± 0.23	14.74 ± 0.74
18% Gluten + 1.2% L-lysine	10	129 ± 4	11.67 ± 2.45	1.43 ± 0.19	10.24 ± 0.59
	100	223 ± 3	20.38 ± 2.53	3.10 ± 0.47	17.28 ± 1.16

[1] Riboflavin supplement was given 6 times per week.
[2] FMN includes FMN + free riboflavin.
[3] Mean ± SEM.

Increasing the dietary riboflavin from 10 to 100 μg significantly increased the concentrations of FAD and total riboflavin in liver of animals fed casein, gluten or gluten supplemented with lysine ($P < 0.01$). The values for combined fractions of free riboflavin and FMN were more significantly increased by the higher level of riboflavin in animals fed casein ($P < 0.01$) than in animals fed gluten or gluten plus lysine ($P < 0.05$).

The protein source had no significant effect on the concentration of FAD or on total riboflavin in liver of animals fed 10 μg riboflavin. The value obtained for free riboflavin plus FMN in liver of animals fed gluten plus 10 μg of riboflavin was greater than that of animals fed casein or gluten plus lysine ($P < 0.01$). This increase in the level of free riboflavin plus FMN in liver of animals fed the gluten diet supplemented with the lower level of riboflavin was caused by a significant interaction between dietary riboflavin and protein ($P < 0.05$). When the higher level of riboflavin was fed, the concentration of FAD and total riboflavin in liver was significantly less in animals fed the diet containing gluten than in animals fed casein ($P < 0.01$). Supplementation of the gluten diet with lysine had no significant effect on any of the riboflavin fractions.

DISCUSSION

Data recorded during this study for animals fed the higher level of riboflavin are consistent with the view that when an unbalanced protein is fed, the amino acid in plasma which is reduced the most corresponds to the most limiting dietary amino acid, and an increase in the supply of the most limiting amino acid may increase the effect of the dietary deficiency of the second most limiting amino acid (5–8).

The feeding of gluten with 100 μg riboflavin resulted in a depression of plasma lysine, the most limiting dietary amino acid, which was markedly greater than that of other essential amino acids, and in a significant increase in threonine, the second most limiting amino acid. Addition of lysine to the gluten diet decreased the concentration of threonine when adequate riboflavin was fed. Conversely, when the gluten diet was supplemented with marginal levels of riboflavin, an interaction between riboflavin and protein occurred and resulted in a less pronounced change in the concentration of plasma lysine with no apparent change in threonine. These changes were observed in animals for which no apparent difference in growth rate occurred. The data suggest that, although the measurement of plasma amino acids may provide a method which may be used to determine limiting amino acids of dietary proteins under controlled conditions, these measurements may not serve as authentic indices of protein nutrition in areas where other dietary nutrients may be marginal or unbalanced.

The ratio of essential to nonessential amino acids for animals fed gluten or gluten plus lysine was lower than that of animals fed casein. The increase in nonessential plasma amino acids in rats fed the gluten diet resulted primarily from an increase of glycine and serine. In rats fed gluten plus lysine a reduction in the levels of serine and glycine in plasma was accompanied by an increase in alanine and proline but the EN ratio was essentially the same as that of rats fed gluten. These results are in agreement with observations reported by Swendseid et al. (8) who reported EN ratios of 1.01 and 0.91 for weanling rats fed for 28 days diets which contained 18% casein. The EN ratios for rats fed 18% gluten was 0.43. These workers also showed that the addition of lysine to gluten did not significantly alter the EN ratio.

In our study the concentration of lysine in plasma of animals fed 18% gluten was greater than the value reported for nonfasted rats by Swendseid et al. (8). This difference may be related to fasting. Morrison et al. (9) reported that fasting increased the concentration of plasma lysine in rats. As determined by analysis, the lysine content of the gluten and of the 18% gluten diet used in this investigation was 1.8 and 0.32%, respectively.

Since studies (10–12) have shown that riboflavin and protein are mutually limiting, the concentration of riboflavin-coenzymes should provide an evaluation of protein synthesis. The effect of dietary protein on riboflavin retention in liver was reviewed by Bro-Rasmussen (13) with the

conclusion that flavoproteins are "in general the most unstable proteins in the body." Inadequate dietary protein results in catabolism of these proteins with increased excretion of riboflavin. Analyses of liver tissue for FAD showed that the addition of lysine to gluten did not significantly affect the concentration of FAD. Except for threonine, the values for essential amino acids in plasma of rats fed gluten plus lysine were comparable to those of animals fed casein. Since supplementation of the gluten diet with lysine did increase FAD somewhat, the increase in isoleucine, methionine and lysine observed in plasma of lysine-supplemented rats may have contributed to FAD retention.

These data indicate that although plasma amino acids may have value in defining protein status, marginal levels of other nutrients may be involved. Variations in riboflavin-coenzymes in tissue suggested that supplementation of wheat gluten with lysine does not completely meet the demands of the tissues for essential amino acids.

LITERATURE CITED

1. Schaefer, A. E. 1963 Nutritional deficiencies in developing countries. J. Amer. Diet. Ass., *42:* 295.
2. Tallan, H. H., S. Moore and W. H. Stein 1954 Studies on the free amino acids and related compounds in the tissues of the cat. J. Biol. Chem., *211:* 927.
3. Bessey, O. A., O. H. Lowry and R. H. Love 1949 The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. J. Biol. Chem., *180:* 755.
4. Snedecor, G. W. 1956 Statistical Methods. The Iowa State College Press, Ames, Iowa.
5. Denton, A. E., and C. A. Elvehjem 1954 Availability of amino acids in vivo. J. Biol. Chem., *206:* 449.
6. Longenecker, J. B., and N. L. Hause 1959 Relationship between plasma amino acids and composition of the ingested protein. Arch. Biochem. Biophys., *84:* 46.
7. Rosenberg, H. R., E. L. Rohdenburg and R. E. Eckert 1960 Supplementation of bread protein with lysine and threonine. J. Nutr., *72:* 423.
8. Swendseid, M. E., J. Villalobos and B. Friedrich 1963 Ratios of essential-to-nonessential amino acids in plasma from rats fed different kinds and amounts of proteins and amino acids. J. Nutr., *80:* 99.
9. Morrison, A. B., J. M. McLaughlan, F. J. Noel and J. A. Campbell 1961 Blood amino acid studies. III. Effects of amount and quality of dietary protein and length of test period on plasma free lysine levels in the rat. Can. J. Biochem. Physiol., *39:* 1681.
10. Czaczkes, J. W., and K. Guggenheim 1946 The influence of diet on the riboflavin metabolism of the rat. J. Biol. Chem., *162:* 267.
11. Sarett, H. P., and W. A. Perlzweig 1943 The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats. J. Nutr., *25:* 173.
12. Wright, L. D., and H. R. Skeggs 1946 Vitamin B-complex studies with diets differing in the level of protein. Proc. Soc. Exp. Biol. Med., *63:* 327.
13. Bro-Rasmussen, F. 1958 The riboflavin requirement of animals and man and associated metabolic relations. II. Relation of requirement to the metabolism of protein and energy. Nutr. Abstr. Rev., *28:* 369.

Mobilization of Liver Vitamin A in Mature Sheep [1,2]

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ABSTRACT Six mature rams averaging 63.3 kg were injected intrajugularly with 268 μCi of 11,12-^{3}H-vitamin A acetate in 4 ml of aqueous 20% Tween 80. The sheep were fed alfalfa hay ad libitum plus 680 g of cracked shelled corn per head daily, supplemented with vitamin A palmitate at a level necessary to maintain liver vitamin A stores. The decline in serum radioactivity postinjection indicated rapid clearance of the injected dose of vitamin A from the blood. The excretory pattern of radioactivity in the feces and urine during 14 days postinjection suggested equilibration of body vitamin A stores. Liver was sampled at 14-day intervals postinjection by aspiration biopsy and analyzed for vitamin A and tritium activity. Vitamin A turnover in the liver was calculated using changes in specific activity units (dpm/μg vitamin A). The half-time of liver vitamin A was 163 ± 48 days and the turnover time was 234 ± 69 days. Using an average liver weight of 1 kg per ram, daily turnover rate of vitamin A was 968 ± 84 μg.

Knowledge of turnover rates of body constituents under varying nutritional, environmental and physiological conditions is pertinent to an evaluation of the nutrient status of the animal. The stores of vitamin A in the liver are frequently used as indices of vitamin A status. Changes in metabolism during aging can be reasonably postulated to change both the need for vitamin A and its metabolism. Little previous attention has been given to the possibility that aging might alter the use of vitamin A stores. Mitchell et al. (1) reported a half-time of 75 days and a turnover time of 108 days for liver vitamin A stores in young ram lambs. The following experiment was conducted to study the turnover of vitamin A stores in the liver of mature rams.

EXPERIMENTAL PROCEDURE

Six mature crossbred rams, 3 years of age or older and averaging 63.3 kg, were injected with 268 μCi of 11,12-^{3}H-vitamin A acetate [3] (specific activity = 213 μCi/mg) and 235,000 IU of unlabeled vitamin A acetate in 4 ml of aqueous 20% polyoxyethylene sorbitan monooleate (Tween 80) into the right jugular vein. The rams were obtained commercially and had been used for two or more breeding seasons. Beginning 1 month before injection, the sheep were fed alfalfa hay ad libitum plus 680 g of cracked shelled corn per sheep daily. To maintain constant liver stores of vitamin A, the corn was supplemented with vitamin A palmitate at a level of twice the NRC (2) recommendation for the first 8 weeks of the experiment and 2.75 times the NRC recommendation for the remainder of the experiment.

Feces and urine were collected daily in metabolism crates for the first 14 days of the experiment. Blood samples were taken by catheter in the left jugular vein at 5, 15, 30 minutes and 1 and 3 hours; and by jugular puncture at 6 hours and 1, 3, 5 and 7 days postinjection. Blood was also sampled at 14-day intervals postinjection. Vitamin A was extracted from serum by the method of Kimble (3) and estimated by the method of Dugan et al. (4).

Fourteen days were allowed after injection for equilibration of liver vitamin A stores before liver biopsy sampling was initiated. Liver samples were taken by the aspiration biopsy technique of Dick (5) at 14-day intervals for 140 days. The liver samples were refrigerated in physiological saline and analyzed in duplicate for vitamin A (4) the following day. All radioactivity determinations were made with a

Received for publication May 12, 1969.

[1] Paper no. 68-5-123, published with the approval of the Director of the Kentucky Agricultural Experiment Station.

[2] Supported in part by Public Health Service Research Grant no. AM 08355 from the National Institute of Arthritis and Metabolic Diseases.

[3] Prepared and donated by Hoffmann-LaRoche Inc., Nutley, New Jersey.

liquid scintillation counter.[4] Radioactivity of the ether extracts of serum, feces and saponified liver samples was determined using 20 ml of the scintillation system described by Mitchell et al. (1). Radioactivity determinations of urine and water extracts of feces were made using the system of Langham et al. (6). The half-time, turnover time and turnover rate of liver vitamin A were calculated as described by Zilversmit (7).

RESULTS AND DISCUSSION

The radioactivity and vitamin A in the ether extracts of serum samples taken at intervals postinjection are presented in table 1. The rapid removal of vitamin A from the blood and concomitant decline in radioactivity throughout the sampling period is consistent with previous studies (1, 8). The radioactivity observed in the urine for the first 14 days after injection is presented in table 2. The urine from four sheep which had received no radioactive material was composited and used in determining background counts. The urinary radioactivity was highest on the first day after injection and tended to remain relatively stable from day 4 throughout the remainder of the collection period. This suggests clearance of the initial dose and stabilization of the relationship between stores and urinary excretion within 4 days. However, stabilization of blood radioactivity values required somewhat longer (table 1).

TABLE 1

Vitamin A and radioactivity in serum of sheep at intervals postinjection with ^{3}H-vitamin A

Time after injection	Vitamin A	Tritium activity
	μg/ml [1]	*dpm/ml* [1]
5 min	0.76±0.06	10,270±1,400
15 min	0.56±0.05	5,000± 360
30 min	0.50±0.04	4,040± 480
1 hr	0.50±0.05	3,800± 390
3 hr	0.58±0.04	4,070± 250
6 hr	0.56±0.06	3,870± 390
1 day	0.53±0.02	2,520± 180
3 days	0.51±0.03	1,410± 280
5 days	0.52±0.04	1,180± 110
7 days	0.51±0.04	890± 120
14 days	0.55±0.04	550± 100
28 days	0.51±0.03	420± 80
42 days	0.50±0.03	330± 50
56 days	0.50±0.04	250± 20
70 days	0.59±0.08	270± 50
84 days	0.55±0.05	200± 40
98 days	0.48±0.07	190± 30
112 days	0.42±0.05	140± 20
126 days	0.33±0.04	127± 20
140 days	0.34±0.10(4)	103± 30(4)

[1] Means± SEM for 6 rams, except on day 140 when only 4 rams were observed.

Total fecal radioactivity per day for the 14-day collection period is also presented in table 2. A 10% aliquot of the daily fecal excretion from each sheep was dried in a forced-air oven at 95°, equilibrated

[4] Packard model 4322, Packard Instrument Company, Inc., Downers Grove, Ill.

TABLE 2

Tritium activity of urine and feces at daily intervals postinjection with ^{3}H-vitamin A

Days after injection	Urine		Feces	
	Tritium activity	Tritium activity	Water extracts	Ether extracts
	10^2 dpm/ml [1]	*10^6 dpm/day*	*10^6 dpm/day*	*10^4 dpm/day*
1	644±153(5)	29.9±6.7	11.5±4.0	69.9±21.8
2	175± 17	11.5±1.3	15.2±7.7	86.3±25.1
3	62± 14	5.6±2.3	16.6±5.1(5)	67.0±18.9
4	48± 7	5.7±1.1	7.7±3.6	50.7±13.3
5	46± 6	3.4±0.8	3.4±0.7	13.2± 6.1
6	57± 6	5.1±1.0	5.7±1.4(5)	36.2±28.8
7	54± 4	3.5±0.7	4.1±1.7(5)	21.5±16.5
8	50± 8	4.0±0.6	5.7±2.3	20.4±19.5
9	49± 2	4.2±0.5	6.3±2.0	1.3± 1.3
10	36± 7	3.9±0.8	4.3±2.1	29.2±27.5
11	48± 4	4.6±1.0	5.0±1.8(5)	18.5±17.6
12	47± 4	4.1±0.5	3.8±1.9	18.4± 5.4
13	34± 6	4.3±0.9	5.5±1.8	33.1±33.1
14	45± 4	3.8±0.7	3.8±1.0	15.0±15.0(5)

[1] Means ± SEM for 6 rams, except where indicated in parentheses.

in air, and ground with a hand grinder. Upon simultaneous extraction of 0.5 g of air-dry feces with 10 ml water and 10 ml petroleum ether, the major portion of the radioactivity was found to be associated with the water-soluble extracts.

The regression of micrograms vitamin A per gram of fresh liver on time showed that the concentration of vitamin A in the liver of each ram did not change significantly ($P > 0.05$). The mean initial value was 229 ± 57 μg/g, and the mean final value was 236 ± 91 μg/g; of the six subjects, only two changed as much as 10% in liver vitamin A concentration (one increasing and one decreasing). The mean value of vitamin A per gram of liver for each individual ram was, therefore, used to calculate specific activity of liver vitamin A (dpm/μg vitamin A). Equations for regression (9) of specific activity on time were developed for each ram (table 3).

TABLE 3

Regression equations used to calculate turnover of liver vitamin A in mature rams

Sheep no.	Regression equation [1]
1	$\log Y = 3.1882 - 0.004651\ X$
2	$\log Y = 2.7805 - 0.002555\ X$
3	$\log Y = 2.8479 - 0.002686\ X$
4	$\log Y = 2.6906 - 0.001804\ X$
5	$\log Y = 2.5919 - 0.000785\ X$
6	$\log Y = 3.1436 - 0.002566\ X$

[1] Y = specific activity of liver vitamin A (dpm/μg vitamin A) and X = days on experiment.

These regression equations were used to determine the half-time and turnover time of liver vitamin A for each individual ram. The half-time of liver vitamin A was 163 ± 48 days (SEM) and the turnover time was 234 ± 69 days (SEM). These times compare with a half-time and a turnover time of 75 and 108 days, respectively, in young ram lambs (1) fed in the same manner. Because of its possible relationship to requirements and both theoretical and actual depletion rates as well as other parameters determining the total dynamics of vitamin A storage and use, the amount of vitamin A represented by this turnover is of significance. An average liver weight of 1 kg per ram was used to calculate turnover rate of liver vitamin A. Turnover rate of liver vitamin A for the mature rams was 968 ± 83 μg/day.

Comparison of this daily turnover with that for young rams (1) requires consideration of differences in liver size (1000 vs 725 g), concentration of vitamin A in the liver (215.4 ± 49.9 vs 84.0 ± 6.6 μg/g) and size of animal (63.3 vs 32.0 kg). Using actual liver weights, linear adjustment of the concentration of vitamin A in the livers of the young rams to that in the mature rams, and the experimentally determined turnover times, the daily turnover of vitamin A per unit of metabolic size ($W_{kg}^{0.75}$) was 43.3 μg for the mature rams and 107.5 μg for the young rams. These data suggest a slower mobilization of liver vitamin A in sheep as the age of the animal increases.

LITERATURE CITED

1. Mitchell, G. E., Jr., C. O. Little, H. B. Sewell and B. W. Hayes 1967 Mobilization of liver vitamin A in sheep. J. Nutr., *91:* 371.
2. National Research Council 1964 Nutrient Requirements of Domestic Animals no. 5. Nutrient Requirements of Sheep. National Academy of Sciences — National Research Council publication 1193, Washington, D. C.
3. Kimble, M. S. 1939 The photoelectric determination of vitamin A and carotene in human plasma. J. Lab. Clin. Med., *24:* 1055.
4. Dugan, R. E., N. A. Frigerio and J. M. Siebert 1964 Colorimetric determination of vitamin A and its derivatives with trifluoroacetic acid. Anal. Chem., *36:* 114.
5. Dick, A. T. 1944 Aspiration biopsy of the liver in sheep. Aust. Vet. J., *20:* 298.
6. Langham, W. H., W. J. Eversole, F. N. Hayes and T. T. Trujillo 1956 Assay of tritium activity in body fluids with use of a liquid scintillation system. J. Lab. Clin. Med., *47:* 819.
7. Zilversmit, D. B. 1960 The design and analysis of isotope experiments. Amer. J. Med., *29:* 832.
8. Hayes, B. W., G. E. Mitchell, Jr., C. O. Little and H. B. Sewell 1967 Turnover of liver vitamin A in steers. J. Anim. Sci., *26:* 855.
9. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.

Undernutrition in Young Miniature Swine [1]

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ABSTRACT A 12-week study, using 9-week old Hormel miniature swine, was conducted to evaluate changes in serum biochemical and hematological parameters, body composition, radiologic determinations and organ weights resulting from protein undernutrition. Each pig was weighed weekly and blood samples were collected biweekly. There was no difference in dietary consumption, calculated on a feed/kilogram body weight/day basis, as a result of feeding the 4% protein diet. At the termination of the study, the protein deficient pigs had lower mean serum total protein, albumin, β-globulin, calcium and inorganic phosphorus concentrations. The albumin/globulin ratio was decreased as a result of feeding the low protein diet. Packed cell volume, hemoglobin concentration, mean cell volume and mean corpuscular hemoglobin were all lower in pigs fed the 4% protein diet than in controls. Total body water, extracellular water, intracellular water and mEq of total potassium, as a function of body weight, were not affected by dietary treatment. The radii of the protein deficient pigs were smaller and the cortex thinner than those of the control pigs which indicated a relative reduction in cortical bone. The effect of protein undernutrition on organ weights, ranking from those least affected to those most affected, was as follows: brain, thyroid, tibia, heart, adrenals, lungs, liver, spleen, gastrocnemius muscle and kidneys.

During a 2-year study of causes of infant deaths in Guatemala, Behar et al. (1) reported that nearly 40% of children 1 to 4 years of age exhibited lesions characteristic of kwashiorkor at the time of death. Cravioto (2) reported that children under 6 months of age at the time of hospital admission for malnutrition (or diseases often secondary to malnutrition) did not exhibit a recovery of mental performance. Wertheimer and Bentor (3) have also implicated malnutrition in cardiovascular problems.

Biochemical changes associated with protein undernutrition must be more completely understood to achieve more definitive treatments for children suffering from this chronic disease. To aid in defining changes associated with undernutrition during prepuberty, an experiment was designed to evaluate changes in serum biochemical and hematological parameters, body composition, radiological determinations and organ weights resulting from protein undernutrition in swine. Swine were chosen as experimental subjects because they resemble humans in many respects (4) and were adaptable for studying the selected parameters.

MATERIALS AND METHODS

Thirteen Hormel miniature swine from two litters were selected for this study which was conducted from July through October, 1968. Each pig was weighed at 9 weeks of age (1 week after weaning) and randomly assigned to a dietary treatment on the basis of weight, litter and sex. Compositions of the diets, which were fed ad libitum for the 12-week experimental period, are presented in table 1. The group of control pigs fed the 16% protein diet was composed of 3 gilts and 3 barrows weighing 6.13 ± 1.60 kg, and the group fed the 4% protein diet was composed of 3 gilts and 4 barrows weighing 6.00 ± 1.42 kg (table 2).

The pigs were housed in two pens in a wooden house with a dirt floor and 2 × 4 m outside runs. Each pig was weighed weekly and the amount of diet given to

Received for publication July 1, 1969.

[1] Supported in part by U. S. Public Health Service Grants FR-00390 and FR-00285.

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each group was recorded weekly. Correlation coefficients, analyses of variance, linear regression equations and standard deviations were calculated according to Steel and Torrie (5).

Blood. Thirteen-milliliter samples were collected, biweekly, from the anterior vena cava. Each animal was fasted 15 hours prior to sample collection between 6 and 8 AM. Two milliliters of the blood, with EDTA-K as the anticoagulant, were used for hematological determinations and the remainder was allowed to clot at room temperature for 2 hours prior to separation of the serum at 0° and 12,000 × *g* for 10 minutes. The serum was frozen and stored at − 10°until analyzed.

Hematological determinations included erythrocyte counts (RBC), red cell size distributions (SD), leukocyte counts (WBC), packed cell volumes (PCV) and hemoglobin concentrations (Hgb). Mean cell volumes (MCV), mean corpuscular hemoglobins (MCH) and mean corpuscular hemoglobin concentrations (MCHC) were calculated. Erythrocyte and leukocyte counts were enumerated using an electronic cell counter and the red cell size distributions were determined using a particle size distribution analyzer plotter.[7] PCV were determined by the microhematocrit method and Hgb were determined using a hemoglobinometer.[8]

Total protein (TP), blood urea nitrogen (BUN), creatinine (Crt), alkaline phosphatase (AP), lactic dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT), calcium (Ca), inorganic phosphorus (IP), chloride (Cl), cholesterol (Chol), total bilirubin (TB) and glucose (Glu) were determined using a modified survey model Sequential Multiple AutoAnalyzer[9] (SMA-

TABLE 1

Composition of diets

Ingredients	Protein in diet	
	16%	4%
	kg/100 kg diet	
Cornstarch	26.2	63.1
Sugar	20.0	20.0
Soybean meal, 50% protein	28.0	7.0
Corn, ground shelled	22.0	5.5
Dicalcium phosphate	2.0	3.0
Calcium carbonate	0.8	0.4
Salt [1]	0.5	0.5
Vitamin-antibiotic mix [2]	0.5	0.5
Digestible energy, kcal/kg	3550	3750
Calcium, %	0.79	0.80
Phosphorus, %	0.60	0.60

[1] Provided 50 ppm Zn in the final mix.
[2] Provided the following: (per kg diet) vitamin A, 4500 IU; vitamin D, 800 IU; riboflavin, 17.6 mg; nicotinic acid, 79 mg; calcium pantothenate, 35.2 mg; choline chloride, 88 mg; vitamin B_{12}, 44 μg; and a 2:2:1 mixture of chlortetracycline–sulfamethiazine–penicillin, 550 mg.

[7] Model B counter and model J plotter, Coulter Electronics, Hialeah, Florida.
[8] Model 231, Instrumentation Laboratory, Watertown, Massachusetts.
[9] Technicon Corporation, Tarrytown, New York.

TABLE 2

Birth, initial and final (12 weeks on test; 21 weeks of age) body weights of the pigs

Pig no.	Litter no.	Sex	Protein in diet	Birth wt	Initial wt	Final wt
			%	*kg*	*kg*	*kg*
477	66	F	16	0.40	3.46	20.2
479	66	F	16	0.82	5.96	30.6
481	66	F	16	0.74	6.88	31.1
483	66	B	16	0.58	5.54	31.3
489	67	B	16	0.78	6.72	39.9
490	67	B	16	0.91	8.24	42.9
			Mean	0.70	6.13	32.7
			SD	0.18	1.60	8.0
478	66	F	4	0.66	5.20	7.5
482	66	B	4	0.60	5.60	10.2
484	66	F	4	0.58	4.24	7.2
485	66	B	4	0.72	6.29	9.3
486	66	B	4	0.74	5.22	7.2
487	67	B	4	0.93	8.62	14.3
488	67	F	4	0.72	6.86	8.6
			Mean	0.71	6.00	9.2
			SD	0.12	1.42	2.5

12/30) (6). Calcium/phosphorus ratios were calculated. Sodium (Na) and potassium (K) concentrations were determined by flame photometry.[10] The protein fractions were separated into albumin (Alb), *alpha*$_1$-globulin (α_1-Glob), *alpha*$_2$-globulin (α_2-Glob), *beta*-globulin (β-Glob) and *gamma*-globulin (γ-Glob) fractions on cellulose polyacetate strips (using the microzone technique), stained with Ponceau S and quantitated with a densitometer.[11] From these values the albumin/globulin (A/G) ratios were calculated.

Body composition. Deuterium oxide, which is nonradioactive and distributes rapidly and uniformly throughout the body, was used to assess indirectly the amount of total body water (TBW). Extracellular water (ECW), measured by sodium thiocyanate dilution technique, included transcellular water and water in red blood cells. Total body water measured by deuterium oxide also included transcellular water and red blood cell water. The difference between TBW and ECW was intracellular water (ICW). Because both TBW and ECW methods measure transcellular water, the estimate of ICW can be expected to be reliable. Since ICW is in the fat-free part of the cell, it was assumed to represent a measure of body cell mass.

An isotonic, injectable solution of deuterium oxide and sodium thiocyanate was used for the indirect determination of TBW and ECW. The sterilized solution, which contained 1.5 mg thiocyanate and 0.995 g deuterium oxide per milliliter of solution, was weighed in a sterilized syringe to the nearest 0.01 mg to provide a dosage of 1.0 ml of solution per kilogram body weight for each animal. After a 5-ml blood sample was drawn from each pig, the appropriate dosage of combined solution was infused intravenously. The empty syringe, which had contained the deuterium oxide-sodium thiocyanate solution, was reweighed to determine the exact amount injected. After 3 hours had elapsed to allow equilibrium of the solutes with the respective water compartments, 5 ml of blood were collected. The two blood samples were allowed to clot, the serum was separated, sealed in ampules, frozen and stored at $-10°$ until analyzed. Deuterium oxide dilution was determined from part of the serum by the falling-drop technique (7) and thiocyanate dilution was determined by spectrophotometry (8). This technique for the estimation of TBW has been verified as accurate in an investigation with pigs when the in vivo measurement of TBW from falling-drop technique was compared with direct analysis of piglets and the mean difference was found to be 0.4% (9).

Another method used to estimate body cell mass was measurement of total body potassium. Total body potassium was calculated from whole body counting of gamma emissions of naturally occurring ^{40}K, using a 2-*pi* liquid scintillation whole body counter (10). The animals weighing less than 10 kg were centered in a 2-tank small animal detector, which positioned their bodies in an area 18 cm in diameter under the detector. Larger animals were immobilized in a special apparatus (11), wheeled under the large 6-tank detector and the detector was lowered to 2 cm from their bodies. The mean of three 5-minute counts was taken as the measurement of the gamma emissions of ^{40}K. A 5-minute count of background was done, before and after each animal was measured, to determine background count rate. To insure absence of interfering materials, the animals were scrubbed with detergent and thoroughly rinsed and dried before their radioactivity was counted.

Bone. The bones were prepared by modifications of a described technique (12). Sections, approximately 2 cm long, were removed from the proximal portion of the diaphysis of the left tibia of each pig at necropsy, and frozen and stored at $-10°$. The bones were scraped free of all adhering tissue and immersed in acetone for 4 days with changes to fresh acetone daily. The bones were then dried to a constant weight in a vacuum oven at 100° for 48 hours. After the fat-free dry weights were obtained, the bones were ashed in a muffle furnace at 600° for 24 hours.

[10] Model 105, Beckman Instruments, Inc., Fullerton, California.

[11] Gelscan, Gelman Instrument Company, Ann Arbor, Michigan.

At necropsy both forelimbs were removed from the body and lateral radiographs taken of each limb with an X-ray machine with nonscreen film at 102 cm distance. The machine was operated at 80 kv and 100 ma for 1/15 second. An aluminum step wedge was placed parallel to the limb near the center of each film. A densitometer [12] was used to determine the density of the central area of the distal epiphysis of the radius on the film. The density of the epiphysis was corrected to g/cm² of aluminum from a plot constructed for each film of the densitometry reading versus the weight per unit area of the aluminum wedge (13). The length of the radius was measured with a dial caliper on the same radiograph and the mean of three readings used. The cortical width of the anterior margin of the radius was measured in the midshaft region at the thickest portion of the compact bone.

Organ weights. At 21 weeks of age each pig was killed by electrocution and exsanguinated. The following tissues and organs were cleanly dissected and weighed: right kidney, left kidney, right adrenal, left adrenal, right lung, left lung, liver, thyroid, heart, brain, left tibia, left gastrocnemius muscle and spleen.

RESULTS

The pigs fed the 16% protein diet gained steadily throughout the duration of the 12-week experimental period (fig. 1); however, the pigs fed the 4% protein diet gained only 3.3 kg during the first 4 weeks and barely maintained their body weight throughout the remainder of the period. The growth rate of the control pigs, from 9 to 21 weeks of age, was greater than previously reported for Hormel miniature swine fed a corn-soybean meal diet (14). Pigs fed the 16% protein diet consumed significantly ($P < 0.01$) more feed, on the basis of daily intake, than did pigs fed the 4% protein diet. When feed intake was calculated on a feed/kilogram body weight per day basis, there was no difference between the two groups.

Blood. The level of serum TP increased for pigs fed the 16% protein diet (fig. 2), whereas the level decreased slightly when the pigs were fed the 4% protein diet.

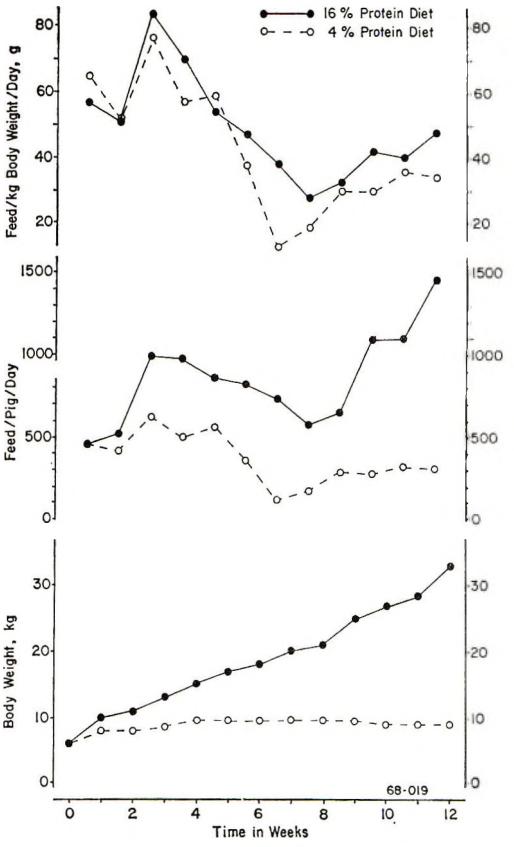

Fig. 1 Body weight and feed intake of pigs fed 4 or 16% protein diets for 12 weeks.

There was a significant ($P < 0.01$) difference between the rates of change of serum TP for the two groups (table 3), as well as a significant ($P < 0.01$) difference between serum TP, of the two groups, after 12 weeks on experiment (table 4). There were no changes in concentrations of BUN or Crt during the experimental period.

Changes in serum Alb concentrations (fig. 3) reflected changes in TP concentrations. Pigs fed the 16% protein diet for 12 weeks had significantly ($P < 0.01$) greater serum Alb levels than did those fed the 4% protein diet (table 4). During the 12-week period, the rate of change in serum Alb concentration was significantly ($P < 0.01$) different for the two groups (table 3). The mean level of serum Alb increased from 1.93 to 2.68 g/100 ml for

[12] Model 501A, Photovolt Corporation, New York, New York.

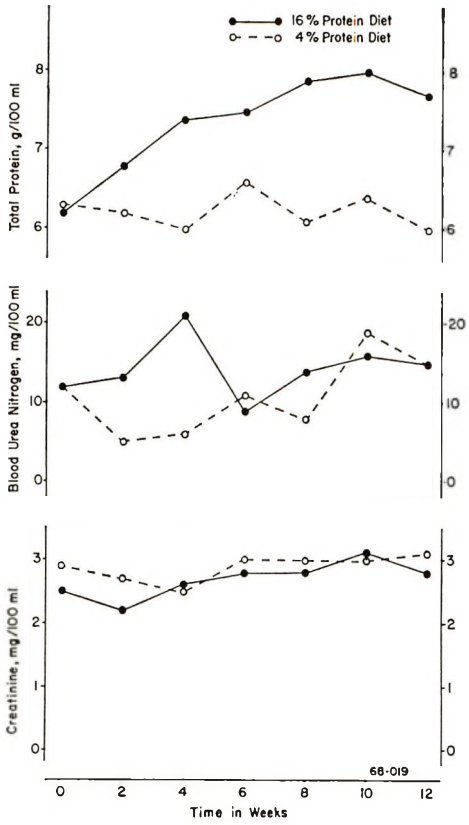

Fig. 2 Total protein, blood urea nitrogen and creatinine in serum of pigs fed 4 or 16% protein diets for 12 weeks.

pigs fed the 16% protein diet, whereas there was a decrease from 2.09 to 1.54 g/100 ml for pigs fed the 4% protein diet. There were no differences in either α_1-Glob or α_2-Glob levels as a result of treatment effect.

The levels of serum β-Glob increased significantly ($P < 0.01$) more rapidly (table 3) in pigs fed the 16% protein diet compared with pigs fed the 4% protein diet. The concentrations of serum γ-Glob, in pigs fed the 4% protein diet, increased significantly ($P < 0.01$) during the first 6 weeks of the study, and then decreased; however, the serum levels in pigs fed the 16% protein diet did not follow the same pattern (fig. 4). The pigs fed the 4% protein diet for 12 weeks exhibited significantly ($P < 0.01$) lower serum A/G ratios than did those fed the 16% protein diet (table 4).

There were no differences in serum AP, LDH or GOT activities as a result of dietary treatment (fig. 5; table 4). Mean serum AP, LDH and GOT activities ranged from 12 to 20 K-AU, 260 to 480 WU and 15 to 28 KU, respectively, during the 12-week experimental period (fig. 5).

During the 12-week period, levels of serum IP decreased significantly ($P < 0.01$) for pigs fed the 4% protein diet; however, there was no change for the control pigs (fig. 6). This resulted in a significant ($P < 0.01$) difference in the rate of change of serum IP concentrations as a result of treatment effect (table 3). Serum calcium levels were significanty ($P < 0.01$) lower for pigs fed the 4% protein diet for 12 weeks (table 4). The Ca/P ratio was significantly ($P < 0.01$) greater for the pigs fed the 4% protein diet than for those fed the 16% protein diet. There were some fluctuations in serum sodium, chloride and potassium concentrations (fig. 7), but there were no changes as a result of dietary effect. Likewise, there were no changes in serum Chol, TB or Glu levels as a result of treatment effect (fig. 8).

Dietary treatment did not affect the RBC, but both PCV and Hgb were significantly ($P < 0.01$) lower (table 4) as a result of feeding the lower protein diet (fig. 9). For pigs fed the 4% protein diet, Hgb did not change during the experimental period while PCV decreased. There was a significant ($P < 0.01$) difference in the rate of change of both PCV and Hgb, during the 12-week period, as a result of treatment effect (table 3). Both PCV and Hgb increased with a concomitant increase in RBC for pigs fed the 16% protein diet during the 12-week experimental period. MCV and MCH (fig. 10; table 4) were decreased as a result of the significant ($P < 0.01$) differences in PCV due to treatment effect. WBC was slightly, but not significantly, increased as a result of feeding the low protein diet. The overall means for the control and protein-deficient pigs were 21.3 and 24.7 $\times$ 10^3 cells/mm^3, respectively.

Body composition. Body composition studies indicated that the pigs fed the 4% protein diet had a slightly greater mean relative ECW (32.7%) compared with control pigs (28.6%) (table 5). Mean values

TABLE 3

Differences between regression coefficients, for selected blood parameters with time, during the 12-week experimental period

Parameter	Protein in diet	N	Regr. coef.	F ratio from covariance test
Body weight	16	42	2.1090	46.77 **
	4	49	0.2191	
Total protein	16	42	0.1372	21.08 **
	4	49	−0.0051	
Albumin	16	42	0.0675	20.13 **
	4	49	−0.0299	
β-Globulin	16	42	0.0199	6.97 **
	4	49	−0.0058	
Albumin/globulin ratio	16	42	0.0084	10.02 **
	4	49	−0.0010	
Calcium/phosphorus ratio	16	42	0.0076	39.94 **
	4	49	0.0283	
Inorganic phosphorus	16	42	−0.0116	12.58 **
	4	49	−0.1666	
Calcium	16	42	0.0610	8.48 **
	4	49	−0.0122	
Packed cell volume	16	42	0.4658	22.56 **
	4	49	−0.2747	
Hemoglobin	16	42	0.2717	28.91 **
	4	49	−0.0171	
Mean cell volume	16	42	−1.0937	4.50 *
	4	49	−1.6791	
Mean corpuscular hemoglobin	16	42	−0.1955	7.16 **
	4	49	−0.4398	

* $P < 0.05$; ** $P < 0.01$.

for percentage ICW for the pigs fed the 4% protein diet were slightly lower (24.8%) than for pigs fed the 16% protein diet (27.3%). The ratio of total body potassium values to body weight was about the same in both groups of pigs.

Bone. The protein-deficient diet markedly affected skeletal development, particularly the formation of compact bone (table 6). The length of the radius was reduced and the diaphysis width-to-length ratio was less in the protein-deficient pigs. The amount of compact bone was further reduced since the ratio of cortical width to length of radius of the treated animals was only 75% of that of the controls. The densitometric values also reflected the reduction in cortical bone. In the protein-deficient animals there was a significant ($P < 0.01$) correlation (0.90) between the width of the cortex and the densitometric reading. The mineral composition of the compact bone was not affected by feeding the low protein diet because the amounts of ash to dry weights of tibial bone did not differ between groups.

Organ weights. The protein-deficient diet significantly ($P < 0.01$) altered the body weights, as well as the weights of all organs and tissues examined in this study (table 7). As expected, the body weight and organ weights of the pigs fed the 4% protein diet were lower. When the weights of control organs were compared with the weights of treated organs, an order of sensitivity was recognized (table 7). The brain, thyroid and wet bone were least affected and the skeletal muscle and visceral organs were most affected. Since most of the weight differences between the two groups can be accounted for in the skeletal muscle and visceral organs, the remaining organs and tissues from animals fed the 4% protein diet accounted for a greater percentage of the body weight.

TABLE 4

Serum biochemical and hematological values [1] for pigs fed different levels of dietary protein for 12 weeks

Parameter	Level of protein in diet	
	16%	4%
Total protein, g/100 ml	7.7[c] ± 0.5	6.0[d] ± 0.4
Albumin, g/100 ml	2.68[c] ± 0.19	1.54[d] ± 0.15
α_1-Globulin, g/100 ml	0.53 ± 0.12	0.30 ± 0.16
α_2-Globulin, g/100 ml	1.90 ± 0.40	1.85 ± 0.18
β-Globulin, g/100 ml	1.38[a] ± 0.25	1.07[b] ± 0.17
γ-Globulin, g/100 ml	1.21 ± 0.25	1.28 ± 0.22
Albumin/globulin ratio	0.55[c] ± 0.09	0.34[d] ± 0.05
Lactic dehydrogenase, WU	366 ± 103	429 ± 66
Alkaline phosphatase, K-AU	18 ± 2	17 ± 3
Glutamic-oxaloacetic transaminase, KU	27 ± 6	22 ± 5
Blood urea nitrogen, mg/100 ml	15 ± 2	15 ± 3
Creatinine, mg/100 ml	2.8 ± 0.2	3.0 ± 0.4
Cholesterol, mg/100 ml	114 ± 16	117 ± 8
Glucose, mg/100 ml	134 ± 12	119 ± 19
Total bilirubin, mg/100 ml	0.2 ± 0.1	0.2 ± 0.1
Sodium, mEq/liter	151 ± 5	148 ± 6
Potassium, mEq/liter	4.8 ± 0.6	5.9 ± 0.5
Chloride, mEq/liter	108 ± 4	109 ± 3
Calcium, mg/100 ml	11.8[c] ± 0.6	11.0[d] ± 0.3
Inorganic phosphorus, mg/100 ml	9.1[c] ± 0.3	6.9[d] ± 0.9
Calcium/phosphorus ratio	1.30[c] ± 0.07	1.61[d] ± 0.17
Erythrocyte count $\times 10^6$, cells/mm^3	8.14 ± 0.49	7.18 ± 0.43
Packed cell volume, %	44.0[c] ± 2.4	34.8[d] ± 1.7
Hemoglobin concentration, g/100 ml	15.4[c] ± 0.8	11.9[d] ± 0.6
Mean cell volume, μ^3	54.1[c] ± 2.8	48.6[d] ± 2.4
Mean corpuscular hemoglobin, pg	19.0[c] ± 1.0	16.6[d] ± 0.7
Mean corpuscular hemoglobin conc., %	35.1 ± 0.5	34.1 ± 0.9
Leukocyte count $\times 10^3$, cells/mm^3	26.7 ± 4.2	30.3 ± 6.4

[1] All values are means ± SD.
[a,b] Means in the same row bearing different superscript letters differ significantly ($P < 0.05$).
[c,d] Means in the same row bearing different superscript letters differ significantly ($P < 0.01$).

DISCUSSION

This experiment was designed to study the effect of feeding a low protein diet upon selected biological parameters in swine. The diets were formulated to simulate those conditions which produce protein deficiency symptoms in humans. Because human undernutrition is not the result of a lack of dietary protein only, no additional vitamins or minerals were added to the low protein diet. As has been previously suggested (15), there is a need to "equate the clinical and pathological conditions in man and experimental animals." Consequently, until there is sufficient information to relate the conditions from one species to another, experiments which have numerous variables must be conducted.

The decreased level of serum TP as a result of protein undernutrition is consistent with the findings of other investigators studying humans (16–19), swine (20, 21), rats (22, 23) and monkeys (24, 25). The difference in serum TP was due primarily to lower absolute amounts of Alb and β-Glob. Pond et al. (21) reported a decrease in relative Alb concentration, but an increase in relative α_2-Glob concentration as a result of feeding conventional swine a 3% casein diet for 9 weeks. Ordy (25) observed a decrease in the absolute concentration of serum Alb as a result of feeding a low protein diet to infant monkeys for 7 weeks. Chandrasakharam et al. (26) reported a decrease in hepatic albumin biosynthesis, as a result of a decreased number of active cells, in young rats fed a protein-free diet. The depletion in serum Alb concentration has been reported to be due to a decreased rate of Alb synthesis (27); the serum Alb half-life is longer in marasmic infants than in normal children.

The pigs used in this study did not develop the diffuse moderate subcutaneous edema reported (20, 28) for pigs fed a low

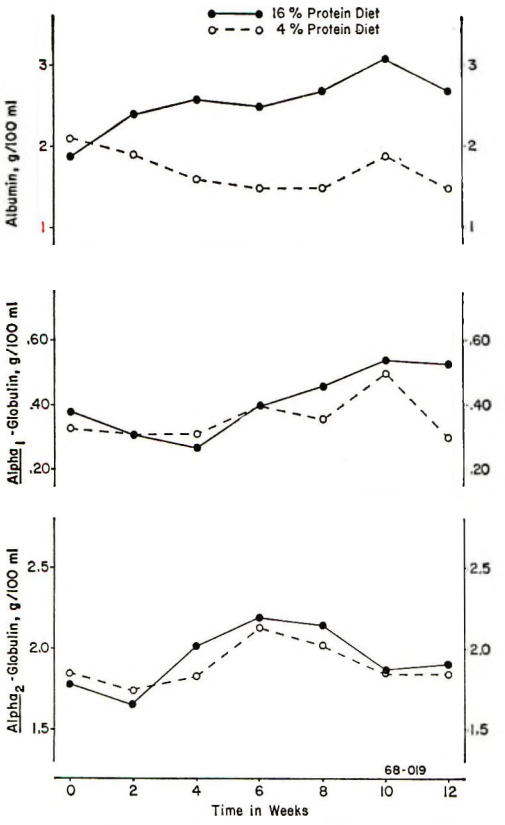

Fig. 3 Serum albumin, *alpha*$_1$-globulin and *alpha*$_2$-globulin concentrations of pigs fed 4 or 16% protein diets for 12 weeks.

protein, wheat gluten-high fat diet. Also, the lack of any difference in TBW, ECW, ICW and mEq total K/kg BW indicated the lack of subcutaneous edema.

In this study, the levels of serum AP activity were not different between the control and protein-deficient pigs. The activities of serum enzymes have been reported to be lowered in some cases of children and animals suffering from undernutrition, but the magnitude of change was not consistent. Perhaps the reason for this is the differences in stage and severity of undernutrition of the experimental subjects. The levels of serum LDH and GOT activities were not affected by the dietary treatment, but were similar to those previously reported for miniature swine (29, 30). The lack of change of the transaminase enzyme activity, as a function of dietary treatment, was indicative of a homeostatic state of overall protein metabolism; however, the relative level of anabolic or catabolic activity may have been markedly affected.

An increase in serum BUN concentration, as a result of treating children with uncomplicated kwashiorkor, was reported by Dean and Schwartz (16). An increase in BUN level may be indicative of an altered protein metabolism due to the greater availability of amino acids present as a consequence of treating the malnourished children with a high protein diet. We did not observe any differences, as a function of dietary treatment, in serum BUN or Crt concentrations or GOT activities; however, this study was conducted during protein undernutrition and not during rehabilitation. Also, the 4% protein diet, in conjunction with the low fat level, may have been sufficient to prevent the changes in pro-

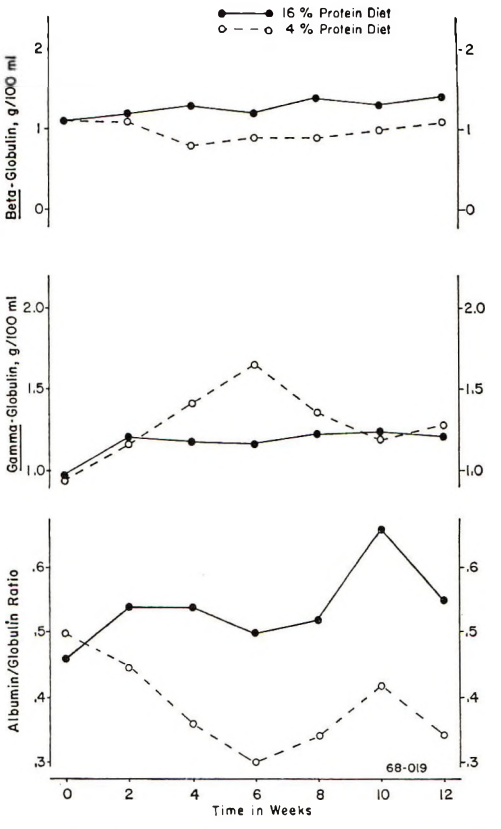

Fig. 4 Serum *beta*-globulin, *gamma*-globulin and albumin/globulin ratio of pigs fed 4 or 16% protein diet for 12 weeks.

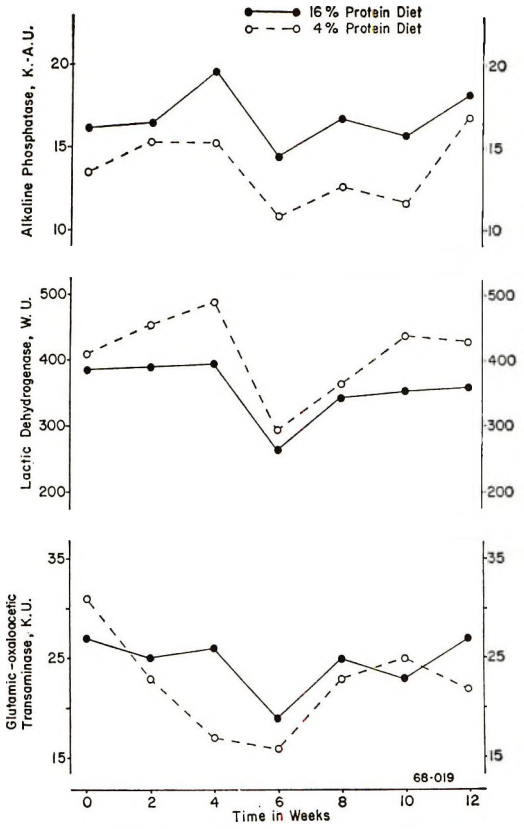

Fig. 5 Alkaline phosphatase (King-Armstrong Units), lactic dehydrogenase (Wacker Units) and glutamic oxaloacetic transaminase (Karmen Units) in serum of pigs fed 4 or 16% protein diets for 12 weeks.

tein metabolism often observed in protein-undernourished children. Lipogenesis, as affected by protein undernutrition, is a process which appears to be a function of diet, protein metabolism and hormonal control; consequently, the elucidation of the controlling mechanism requires the production of an uncomplicated protein-undernutrition syndrome in experimental animals.

The decreased serum IP level, as a result of feeding the low protein diet, was consistent with the previously reported (31) findings for Ethiopian children suffering from advanced protein malnutrition. This was probably not a function of dietary intake of phosphorus since both diets contained 0.6% phosphorus and the consumption of feed/kilogram body weight per day was not different between the control and protein-deficient pigs. The level of serum IP for the control pigs was similar to that previously reported for miniature swine (29, 32).

The concentrations of serum sodium and chloride were not affected by dietary treatment. Many investigators have reported changes in serum electrolyte concentrations of children suffering from protein undernutrition, with most of the subjects exhibiting edema; however, edema was not present in our pigs fed the low protein diet. After 12 weeks, the pigs receiving the 4% protein diet had significantly ($P < 0.01$) less serum potassium. The difference appeared to be an artifact and demonstrated the importance of serial sampling. Garrow (33) reported a loss of brain potassium in severely malnourished

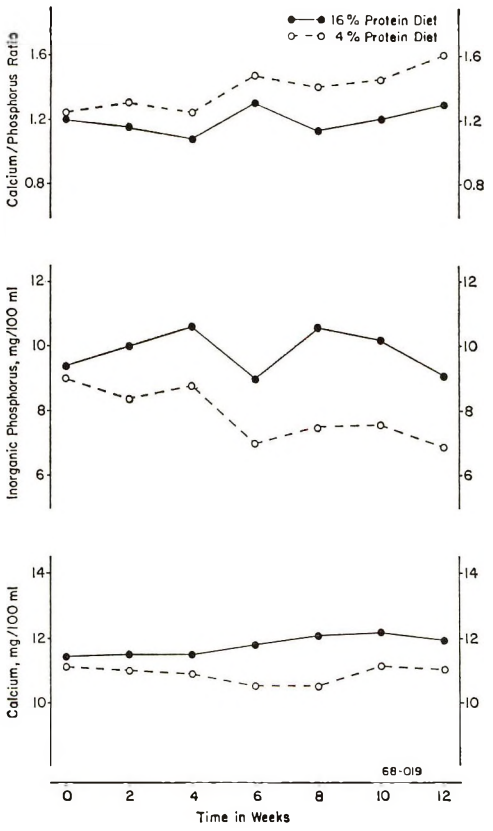

Fig. 6 Calcium, inorganic phosphorus and calcium/phosphorus ratio of pigs fed 4 or 16% protein diets for 12 weeks.

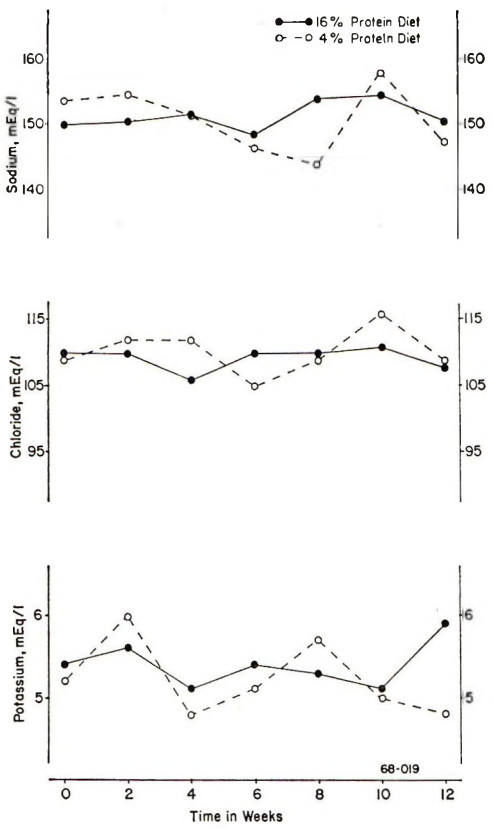

Fig. 7 Sodium, chloride and potassium concentrations in serum from pigs fed 4 or 16% protein diets for 12 weeks.

children. He suggested that the loss of potassium from tissues was not a result of deficiency in dietary potassium, but that it was secondary to the metabolic alterations associated with protein malnutrition. A loss of muscle potassium, during infantile malnutrition, was reported by Smith and Waterlow (34). They measured both exchangeable body potassium and serum potassium concentrations, and concluded that serum potassium level did not reflect the extent of the body potassium loss.

The level of serum Chol, which was comparable to that previously reported (29, 32), was not affected by dietary treatment. There have been reports of protein-calorie undernutrition affecting liver phospholipid levels (35), liver lipogenesis (3), brain lipid levels (36) and concentrations of serum Chol (16, 31, 37). Heidenreich et al. (38) reported a positive correlation between serum Chol concentration and average daily gain of growing swine; they also noted a negative correlation between serum Chol concentration and muscle mass.

Hematologic values for the control pigs were similar to those previously reported for miniature swine (30, 32). The pigs fed the 4% protein diet exhibited a reduction in both PCV and Hgb concentrations; however, because MCHC was not affected by dietary treatment, the pigs were probably not suffering from iron-deficiency anemia. Lowered Hgb has been reported for Ugandese (39) and Ethiopian (31) children with kwashiorkor. Lowrey et al. (20) noted decreased PCV and Hgb concentrations as a result of feeding low protein diets to swine. The presence of iron-deficiency anemia, in children suffering from kwashiorkor, has been an inconsistent finding.

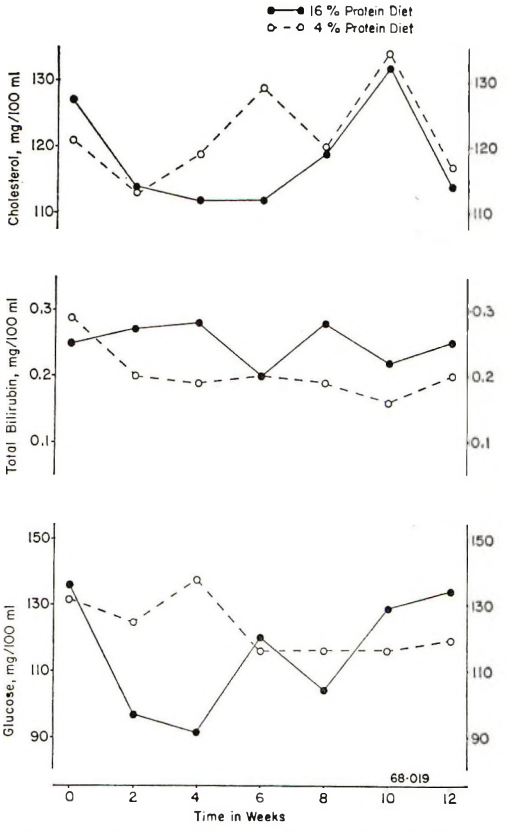

Fig. 8 Cholesterol, total bilirubin and glucose concentrations in serum from pigs fed 4 or 16% protein diets for 12 weeks.

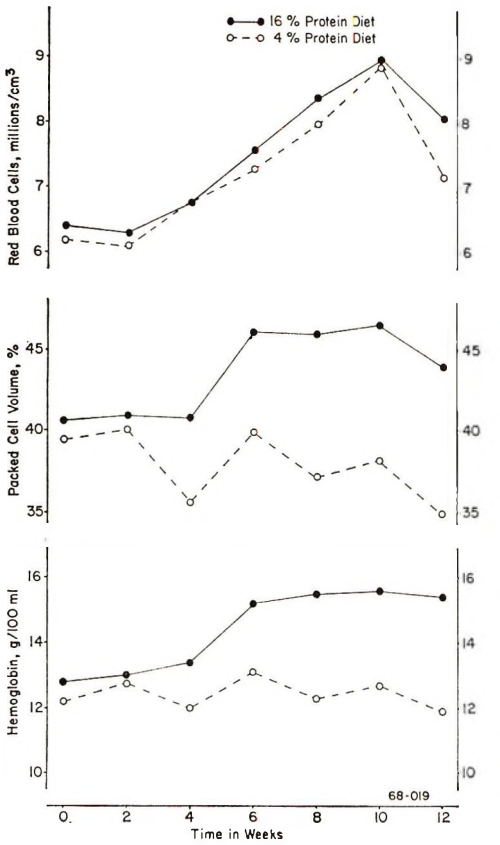

Fig. 9 Erythrocyte count, packed cell volume and hemoglobin concentration in blood from pigs fed 4 or 16% protein diets for 12 weeks.

In the pigs studied here, compartment values did show slight total body water changes. The ECW was slightly increased and ICW was slightly decreased as a result of dietary treatment; however, no conclusions could be drawn from the limited number of pigs. Total body potassium values appeared to be individually variable. Judging from the fact that the intracellular water was lower in the pigs fed the 4% protein diet, one would expect total body potassium to be lower also, but mean potassium per kilogram body weight was not lower. However, a 1-week period of time elapsed between the body water and the total potassium analyses. Compositional changes may have taken place in that time, making a direct comparison difficult.

Several investigators have published data revealing changes in body water compartments in malnourished children. Flynn et al. (40, 41) observed that TBW and ECW

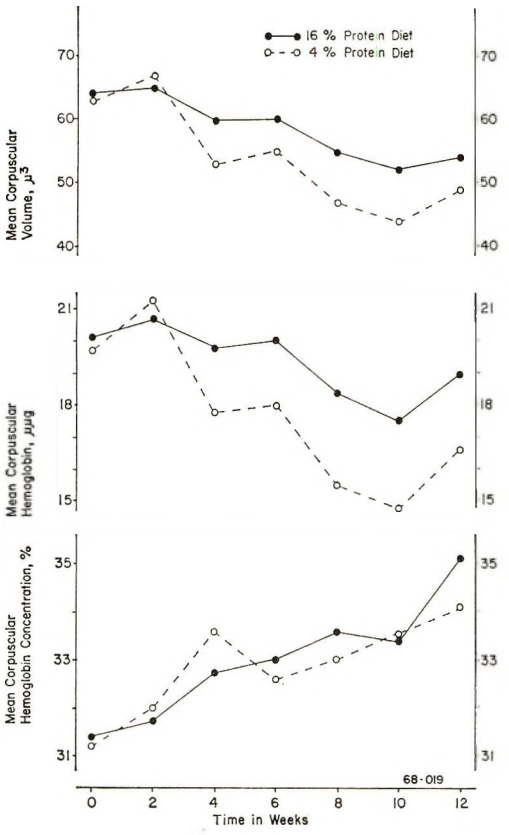

Fig. 10 Mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration in blood from pigs fed 4 or 16% protein diets for 12 weeks.

TABLE 5

Body water and potassium values [1] of pigs fed 4% or 16% protein diets for 11 weeks

Parameter [2]	Protein in diet	
	16%	4%
TBW/BW	56.0 ± 7.0	57.8 ± 6.6
ECW/BW	28.6 ± 3.2	32.7 ± 3.9
ICW/BW	27.3 ± 2.5	24.8 ± 3.9
mEq total K	1781 [c] ± 245	536 [d] ± 88
mEq total K/BW	54.9 ± 7.4	54.2 ± 11.2

[1] All values are means ± SD.
[2] BW = Body weight; TBW = Total body water; ECW = Extracellular water; ICW = Intracellular water.
[c,d] Means in the same row bearing different superscript letters differ significantly ($P < 0.01$).

TABLE 6

Means and standard deviation (±SD) for radiographic measurements of radii of 21-week-old miniature swine fed 16% or 4% protein diets for 12 weeks

Parameter	Protein in diet	
	16%	4%
Cortical width, cm	0.261 [c] ± 0.025	0.119 [d] ± 0.025
Density, g/cm²	2.14 [c] ± 0.17	1.36 [d] ± 0.17
Radius length, cm	8.716 [c] ± 0.356	7.090 [d] ± 0.356
Diaphysis width, cm	0.981 [c] ± 0.059	0.748 [d] ± 0.055
Cortex width × 10/radius length	0.304 [c] ± 0.022	0.237 [d] ± 0.014
Radius width/radius length	0.112 [c] ± 0.005	0.105 [d] ± 0.005

[c,d] Means in the same row bearing different superscript letters differ significantly ($P < 0.01$).

TABLE 7

Effect of level of dietary protein upon organ weights [1] of pigs fed for 12 weeks

Organ	Level of protein in diet		Control wt/ treated wt
	16%	4%	
	g	g	
Kidneys	125.0 [c] ± 29.0	35.6 [d] ± 9.5	3.51
Gastrocnemius muscles	22.63 [c] ± 3.59	6.78 [d] ± 1.26	3.34
Spleen	47.7 [c] ± 12.5	14.6 [d] ± 4.7	3.27
Liver	656 [c] ± 200	215 [d] ± 69	3.05
Lungs	187.6 [c] ± 28.9	67.8 [d] ± 9.9	2.77
Adrenals	2.20 [c] ± 0.74	0.84 [d] ± 0.15	2.62
Heart	123.5 [c] ± 26.2	51.1 [d] ± 11.6	2.42
Tibia	61.2 [c] ± 9.4	27.9 [d] ± 5.0	2.19
Thyroid	2.02 [c] ± 0.44	0.93 [d] ± 0.16	2.17
Brain	76.0 [a] ± 4.9	67.6 [b] ± 6.0	1.12

[1] All values are means ± SD.
[a,b] Means in the same row bearing different superscript letters differ significantly ($P < 0.05$).
[c,d] Means in the same row bearing different superscript letters differ significantly ($P < 0.01$).

were increased in undernourished children compared with normal children of the same age. Brinkman et al. (42) and Smith (43) noted very high TBW values in children with extreme undernutrition, and Brinkman et al. (42) reported increased ECW values. Kwashiorkor children have been reported to be potassium poor (34).

The compact bone found in the protein-deficient pigs appears to have the same degree of mineralization as in the control animals since the ash-to-dry-weight ratios are similar. It has been suggested (44) that compact bone in severely undernourished growing pigs might be overcalcified, because of the radiographic appearance of "cement lines" in the bone of stunted pigs. However, this characteristic appearance was not demonstrated in this study, possibly because of the shorter duration of the experiment.

The reduced cortical width of the protein-deficient animals may partly reflect the reduced growth rate. However, the decreased cortical width-to-bone-length ratios may indicate a limitation of matrix formation with restricted protein intake. The ratio of cortex to bone length for the control animals when they were at the weight of the treated group is not known.

The densitometer readings, although made over an area of both trabecular and lamellar bone, were proportional to the width of the cortex. Both these parameters indicate a probable reduction in mineral content of the whole bone, because a direct relationship has been shown by other investigators (45) between the area of the cortical layer and the mass of bone ash per milliliter of whole bone. These findings would satisfy the description of osteoporosis by Albright (46) as a disturbance of formation of the organic matrix. The reduced bone development in these animals appeared to be essentially a lack of com-

pact bone which has been reported to be characteristic of kwashiorkor (47).

The weights of some organs, such as the kidneys, were more severely affected by the protein-deficient diet than the weights of others, such as the brain. There was a marked difference between the effect of the altered diet on skeletal muscles and bones. The skeletal muscles were severely affected by the diet, whereas the bones were less severely affected. The comparison of organ weights reported by Brown and Guthrie (48) falls into the same order of sensitivity as reported here.

The mechanisms associated with altered metabolic processes in protein undernutrition appear to be complex; therefore, the experiments to study these phenomena must be designed to utilize multivariate analyses. The use of an animal, such as the miniature pig, which enables investigators to study numerous selected parameters is paramount to meaningful experimentation. As has been demonstrated in this study, as well as in previous studies, the pig can be utilized as an experimental model to study metabolic changes which have been observed in other species.

ACKNOWLEDGMENTS

Acknowledgment is extended to Dr. D. P. Hutcheson for statistical consultation and Dr. P. D. Garrett for assistance in preparing the illustrations.

LITERATURE CITED

1. Behar, M., W. Ascoli and N. S. Scrimshaw 1958 An investigation into the causes of death in children in four rural communities in Guatemala. WHO Bull., *19:* 1093.
2. Cravioto, J. 1963 Application of newer knowledge of nutrition on physical and mental growth and development. Amer. J. Public Health Nat. Health, *53:* 1803.
3. Wertheimer, H. E., and V. Bentor 1965 Lipogenesis in undernutrition. Israel J. Med. Sci., *1:* 361.
4. Bustad, L. K., and R. O. McClellan 1966 Swine in Biomedical Research. Frayn, Seattle, Washington.
5. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York.
6. Tumbleson, M. E. 1969 Modification of Sequential Multiple AutoAnalyzer (SMA-12/30) for use in animal research studies. Clin. Biochem., *2:* 357.
7. Schloerb, P. R., B. J. Friis-Hansen, I. S. Edelman, A. K. Solomon and F. D. Moore 1960 The measurement of total body water in the human subject by deuterium oxide dilution. J. Clin. Invest., *29:* 1296.
8. Doxiadis, S. A., and D. Gairdner 1948 The estimation of extracellular fluid volume by the thiocyanate method in children and adults. Clin. Sci., *6:* 259.
9. Flynn, M. A., F. M. Hanna, C. H. Long, R. Y. Asfour, R. N. Lutz and S. E. Zobrisky 1968 Deuterium oxide dilution as a predictor of body composition in children and pigs. In: Body Composition in Animals and Man. National Academy of Sciences – National Research Council, publ. 1958. Washington, D. C., p. 480.
10. Tumbleson, M. E., A. R. Dommert, R. B. Wescott and W. J. Coffman 1968 Effect of administration route upon iron-59 retention in miniature swine as measured in a 2-*pi* liquid scintillation whole body counter. J. Anim. Sci., *27:* 83.
11. Tumbleson, M. E., A. R. Dommert and C. C. Middleton 1968 Techniques for handling miniature swine for laboratory procedures. Lab. Anim. Care, *18:* 584.
12. Trotter, M., and R. R. Peterson 1955 Ash weight of human skeletons in per cent of their dry fat-free weight. Anat. Rec., *123:* 341.
13. Omnell, K. 1957 Quantitative roentgenologic studies on changes in mineral content of bone in vivo. Acta Radiol. Suppl., *148:* 71.
14. Tumbleson, M. E., C. C. Middleton, O. W. Tinsley and D. P. Hutcheson 1969 Body weights and measurements of Hormel miniature swine from birth to nine months of age. Lab. Anim. Care, *19:* 596.
15. Platt, B. S., C. R. C. Heard and R. J. C. Stewart 1964 Experimental protein-calorie deficiency. In: Mammalian Protein Metabolism, vol. II. Academic Press, New York, p. 445.
16. Dean, R. F. A., and R. Schwartz 1953 The serum chemistry in uncomplicated kwashiorkor. Brit. J. Nutr., *7:* 131.
17. Baptist, N. G., C. C. de Silva and M. A. Sideek 1959 Electrophoretic pattern of plasma proteins in clinically diagnosed cases of kwashiorkor (protein malnutrition) and undernutrition (marasmus) among Ceylonese children. Brit. J. Nutr., *13:* 488.
18. Edozien, J. C., and M. E. Obasi 1965 Protein and amino acid metabolism in kwashiorkor. Clin. Sci., *29:* 1.
19. Mukherjee, K. L. 1967 Classification of protein-calorie undernutrition in children. Arch. Dis. Child., *42:* 647.
20. Lowrey, R. S., W. G. Pond, R. H. Barnes, L. Krook and J. K. Loosli 1962 Influence of caloric level and protein quality on the manifestations of protein deficiency in the young pig. J. Nutr., *78:* 245.
21. Pond, W. G., R. H. Barnes, R. B. Bradfield, E. Kwong and L. Krook 1965 Effect of dietary energy intake on protein deficiency symptoms and body composition of baby pigs fed equalized but suboptimal amounts of protein. J. Nutr., *85:* 57.

22. Kirsch, R. E., J. F. Brock and S. J. Saunders 1968 Experimental protein-calorie malnutrition. Amer. J. Clin. Nutr., *21:* 820.
23. Waterlow, J. C., and J. M. L. Stephen 1968 The effect of low protein diets on the turnover rates of serum, liver and muscle proteins in the rat, measured by continuous infusion of L-(^{14}C)-lysine. Clin. Sci., *35:* 287.
24. Deo, M. G., and V. Ramalingaswami 1960 Production of periportal fatty infiltration of the liver in the Rhesus monkey by a protein-deficient diet. Lab. Invest., *9:* 319.
25. Ordy, J. M., T. Samorajski, R. R. Zimmerman and P. M. Rady 1966 Effects of postnatal protein deficiency on weight gain, serum proteins, enzymes, cholesterol, and liver ultrastructure in a subhuman primate (*Macaca mulatta*). Amer. J. Path., *48:* 769.
26. Chandrasakharam, N., A. Fleck and H. N. Munro 1967 Albumin content of rat hepatic cells at different levels of protein intake. J. Nutr., *92:* 497.
27. Donoso, G., O. Brunser and F. Monckeberg 1965 Metabolism of serum albumin in marasmic infants. J. Pediat., *67:* 306.
28. Pond, W. G., R. H. Barnes, I. Reid, L. Krook, E. Kwong and A. U. Moore 1966 Protein deficiency in baby pigs. In: Swine in Biomedical Research. Frayn, Seattle, Washington, p. 213.
29. Tumbleson, M. E., C. C. Middleton, O. W. Tinsley and D. P. Hutcheson 1969 Serum biochemic and hematologic parameters of Hormel miniature swine from four to nine months of age. Lab. Anim. Care, *19:* 345.
30. Tegeris, A. S., F. L. Earl and J. M. Curtis 1966 Normal hematological and biochemical parameters of young miniature swine. In: Swine in Biomedical Research. Frayn, Seattle, Washington, p. 575.
31. Bjornesjo, K. B., M. Belew and B. Zaar 1966 Biochemical study of advanced protein malnutrition in Ethiopia. Scand. J. Clin. Lab. Invest., *18:* 591.
32. McClellan, R. O., G. S. Vogt and H. A. Ragan 1966 Age-related changes in hematological and serum biochemical parameters in miniature swine. In: Swine in Biomedical Research. Frayn, Seattle, Washington, p. 597.
33. Garrow, J. S. 1967 Loss of brain potassium in kwashiorkor. Lancet, *1:* 643.
34. Smith, R., and J. C. Waterlow 1960 Total exchangeable potassium in infantile malnutrition. Lancet, *1:* 147.
35. Chatterjee, K. K., and K. L. Mukherjee 1968 Phospholipids of the liver in children suffering from protein-calorie undernutrition. Brit. J. Nutr., *22:* 145.
36. Culley, W. J., and R. O. Lineberger 1968 Effect of undernutrition on the size and composition of the rat brain. J. Nutr., *96:* 375.
37. Gillman, J., C. Gilbert and N. Savage 1962 Endocrine control of the fatty liver and serum lipids in baboons with special reference to the disorder of lipid metabolism in kwashiorkor. Metabolism, *11:* 800.
38. Heidenreich, C. J., V. A. Garwood and R. B. Harrington 1964 Swine growth and composition as associated with total serum cholesterol. J. Anim. Sci., *23:* 496.
39. Wharton, B. A., G. R. Howells and R. A. McCance 1967 Cardiac failure in kwashiorkor. Lancet, *2:* 384.
40. Flynn, M. A., F. M. Hanna and R. N. Lutz 1967 Estimation of body water compartments of preschool children. I. Normal children. Amer. J. Clin. Nutr., *20:* 1125.
41. Flynn, M. A., F. M. Hanna, R. Y. Asfour and R. N. Lutz 1967 Estimation of body water compartments of preschool children. II. Undernourished children. Amer. J. Clin. Nutr., *20:* 1129.
42. Brinkman, G. L., M. D. Bowie, B. Friis-Hansen and J. D. L. Hansen 1965 Body water composition in kwashiorkor before and after loss of edema. Pediatrics, *36:* 94.
43. Smith, R. 1960 Total body water in malnourished infants. Clin. Sci., *19:* 275.
44. Dickerson, J. W. T., and R. A. McCance 1961 Severe undernutrition in growing and adult animals. 8. The dimensions and chemistry of the long bones. Brit. J. Nutr., *15:* 567.
45. Virtama, P., and H. Mahonen 1960 Thickness of the cortical layer as an estimate of mineral content of human finger bones. Brit. J. Radiol., *33:* 60.
46. Albright, F. 1947 Osteoporosis. Ann. Intern. Med., *27:* 861.
47. Garn, S. M., C. G. Rohmann and F. N. Silverman 1967 Radiographic standards for postnatal ossification and tooth calcification. Med. Radiogr. Photogr., *43:* 45.
48. Brown, M. L., and H. A. Guthrie 1968 Effect of severe undernutrition in early life upon body and organ weights in adult rats. Growth, *32:* 143.

A Histochemical Study of Enzyme Changes and Ultrastructure of the Jejunal Mucosa in Protein-depleted Rats [1]

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ABSTRACT Histochemical estimation of selected brushborder, mitochondrial, lysosomal and microsomal enzymes, and observations on the ultrastructure were made on the jejunal mucosa of young rats fed a low protein diet for 10 weeks. The ultrastructure of the microvilli was not altered nor were there changes in the brushborder enzymes examined. Acid phosphatase and succinic dehydrogenase were significantly reduced, although the mitochondria appeared normal in fine structure. Glucose-6 phosphatase actvity was unchanged, and this correlated with the maintenance of the rough endoplasmic reticulum. The significance of these observations is discussed in relation to malabsorption which has been observed in clinical protein-calorie malnutrition.

Intestinal atrophy and the malabsorption of nutrients occur in children suffering with kwashiorkor (1, 2) as well as in adults with protein-calorie malnutrition (3, 4). These observations have stimulated research on the functional and structural alterations of small intestine in protein-depleted animals (5–8). Histological studies reveal a normal appearance of epithelial cells but a shortening of the villi and a decrease in the number of cells in the crypts. The mechanism of these morphological changes of the villi has been investigated (9, 10), but few enzymatic and ultrastructural studies of jejunal mucosa in protein depletion have been made (11–13). Histochemical studies have proved useful in extending the significance of biochemical observations. Therefore, the present study was conducted to examine histochemically the effect of protein depletion in rats on some enzymatic changes in the absorptive cells of jejunal mucosa and to relate these changes to observations made on the fine structure of the jejunal cells.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain,[3] weighing 154 g initially, were used in the studies. Following their arrival in the animal quarters, they were fed a commercial laboratory diet for 2 days before random allocation between the two experimental diets. The diets were fed ad libitum; their composition is given in table 1.

The purpose of the present investigation was to study the effect of feeding a low protein diet for a 10-week period. A preliminary report (14) has already shown that, with rats of a slightly lower initial body weight than used in the present experiment, the low protein diet results in the development of a number of clinical and biochemical changes which parallel those observed in children suffering with kwashiorkor.

Three rats from each dietary group were killed after 5 weeks on the experiment, and six from each group after 10 weeks. While the animals were anesthetized (sodium pentobarbital, 5 mg/100 g body wt, given by intraperitoneal injection) blood was drawn from the posterior vena cava for serum total protein estimation (15). Specimens were then quickly taken from the jejunum for histological and histochemical observation and for the electron

Received for publication July 1, 1969.

[1] Financial aid provided in part by Grant no. 386 from the Nutrition Foundation, Inc., New York, and in part by Contract no. DA-49-193-MD-2560 with the U.S. Army Medical Research and Development Command. This is publication no. 1496 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

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TABLE 1

Composition of adequate and low protein diets used for studying the effects of protein depletion on rat jejunal mucosa

Ingredient	Adequate	Low
	% of diet	
Lactalbumin [1]	18.0	0.5
Cornstarch	44.2	55.9
Sucrose	22.1	27.9
Cottonseed oil [2]	10.0	10.0
Vitamin mix [3]	0.5	0.5
Mineral mix [3]	5.0	5.0
Choline [4]	0.2	0.2

[1] Purchased from General Biochemicals, Chagrin Falls, Ohio.
[2] Wesson Oil, Wesson Oil Sales Company, Fullerton, California.
[3] Composition described by Rogers and Harper (32).
[4] Added to the diet as an aqueous solution, containing 1 g choline·HCl/5 ml.

microscopic observations. Processing and evaluation of the tissues were carried out stimultaneously in well-nourished and protein-depleted rats.

The enzymes examined in this study and histochemical methods were as follows: a) alkaline phosphatase (16) and adenosine triphosphatase (17) were taken to represent brushborder enzymes; b) acid phosphatase (18) as a representative lysosomal enzyme; c) succinic dehydrogenase (19) for evaluation of mitochondrial activity; d) glucose-6 phosphatase as an example of a microsomal enzyme (20, 21).

For the ultrastructural study the tissue was fixed in glutaraldehyde and postfixed in osmium tetroxide (22). An LKBI ultratome was used for preparing thin sections, which were then stained with uranyl acetate and lead oxide (23) before examination with the aid of a Phillips EM 200 electron microscope.

RESULTS

Rats fed the low protein diet lost weight progressively, and their serum total protein concentrations declined (table 2). General body wasting was a prominent feature in the present study.

Figures 1 and 2 compare the typical histological findings with jejunal mucosa of control and protein-depleted rats after 10 weeks on the experimental diets. The villi were stunted and there were fewer Lieberkühn's crypts in protein-depleted rats, but there were no apparent changes in the morphology of the absorptive epithelial cells in the protein-depleted rats, and the lamina propria did not show any significant inflammatory cellular infiltration.

Fig. 1 Section of the jejunum from a control rat showing normal morphology. Note height of intestinal villi and numerous crypts of Lieberkühn at base of villi. Hematoxylin and eosin. × 100.

Fig. 2 Section of the jejunum comparable to that shown in figure 1 obtained from a protein-depleted rat at 10 weeks. Note shortened intestinal villi and decreased number of crypts of Lieberkühn. Hematoxylin and eosin. × 100.

Fig. 3 Section of jejunum from control rat stained for succinic dehydrogenase. The epithelial cells stain intensely. Nitro BT method for succinic dehydrogenase. × 100.

Fig. 4 Section of jejunum from a protein-depleted rat taken from an area comparable to that shown in figure 3. Note absence of stainable material denoting a sharp decrease in enzymatic activity in the protein-depleted animal. Nitro BT method for succinic dehydrogenase. × 100.

Fig. 5 Section of jejunum from a control rat stained for presence of acid phosphatase. Note large amount of enzyme localized at luminal border of the epithelial cells of the villus. Acid phosphatase stain. × 100.

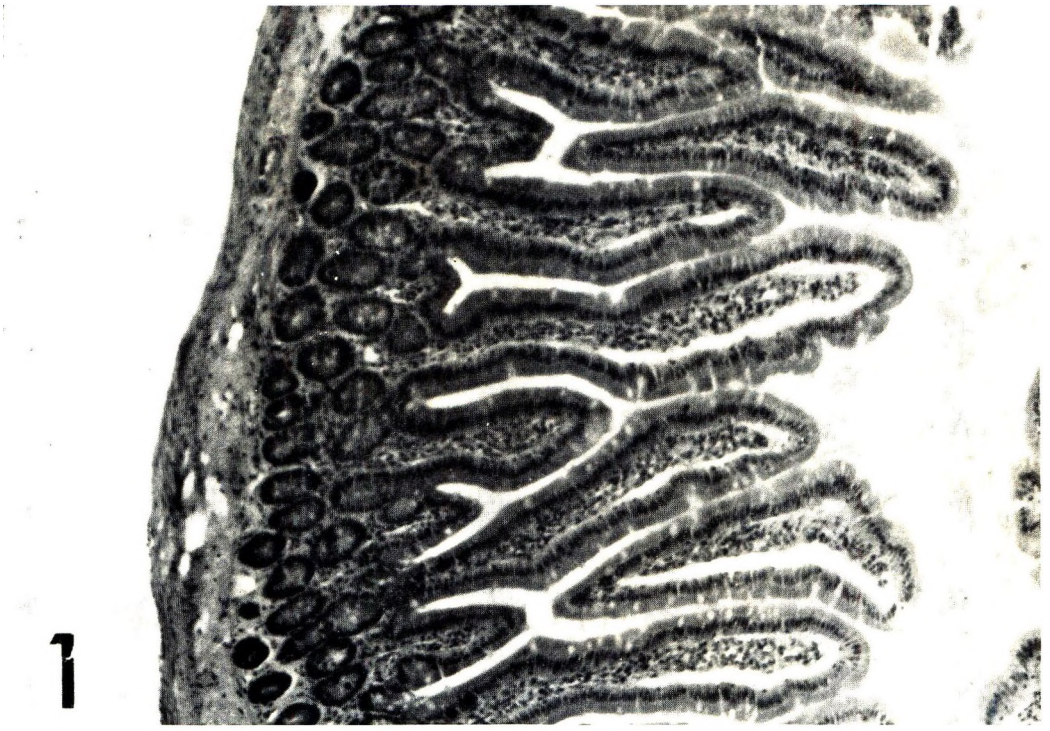
Fig. 6 Section of jejunum from a protein-depleted rat stained for presence of acid phosphatase. Note decrease in amount of enzyme as a result of protein depletion. Acid phosphatase stain. × 100.

TABLE 2

Body weights and serum total protein of rats fed the adequate and low protein diets for 10 weeks [1]

Group	Body weight		Total protein	
	Adequate	Low	Adequate	Low
	g		*g/100 ml*	
5 weeks [2]	348	100	7.1	4.7
10 weeks [3]	419±27	84±5	6.6±0.5	3.6±0.3

[1] Initial weight 154 ± 3 g (SEM).
[2] Mean values for three rats in each group.
[3] Mean ± SD for six rats in each group.



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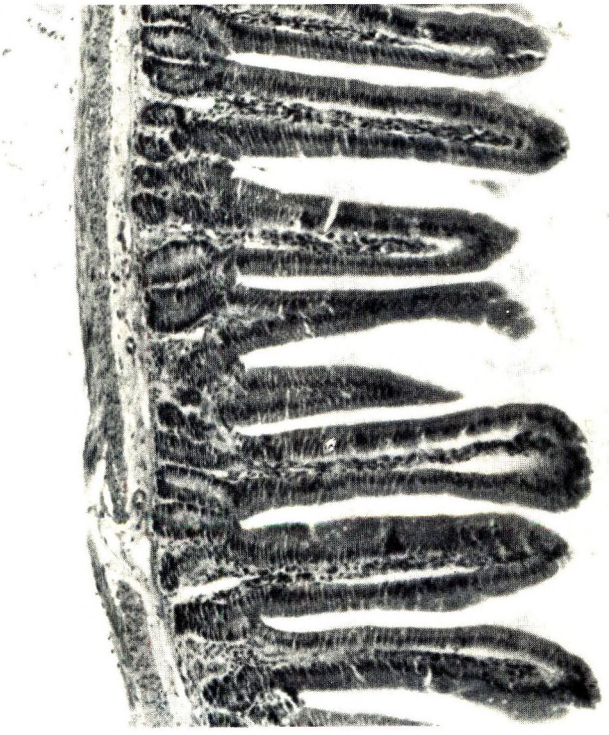

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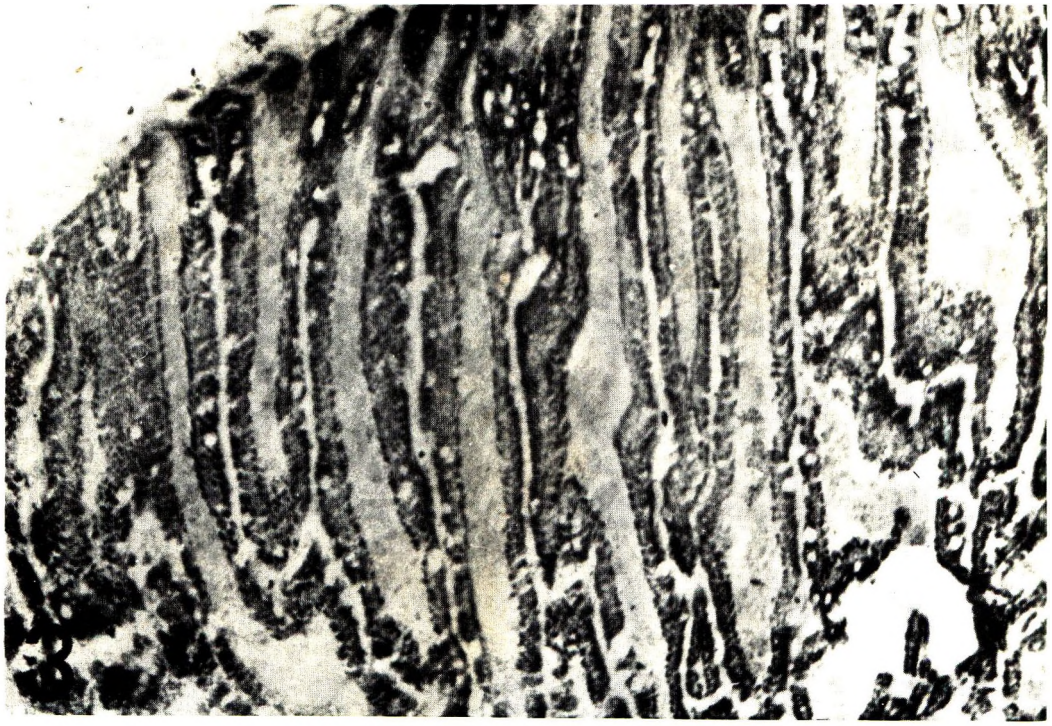

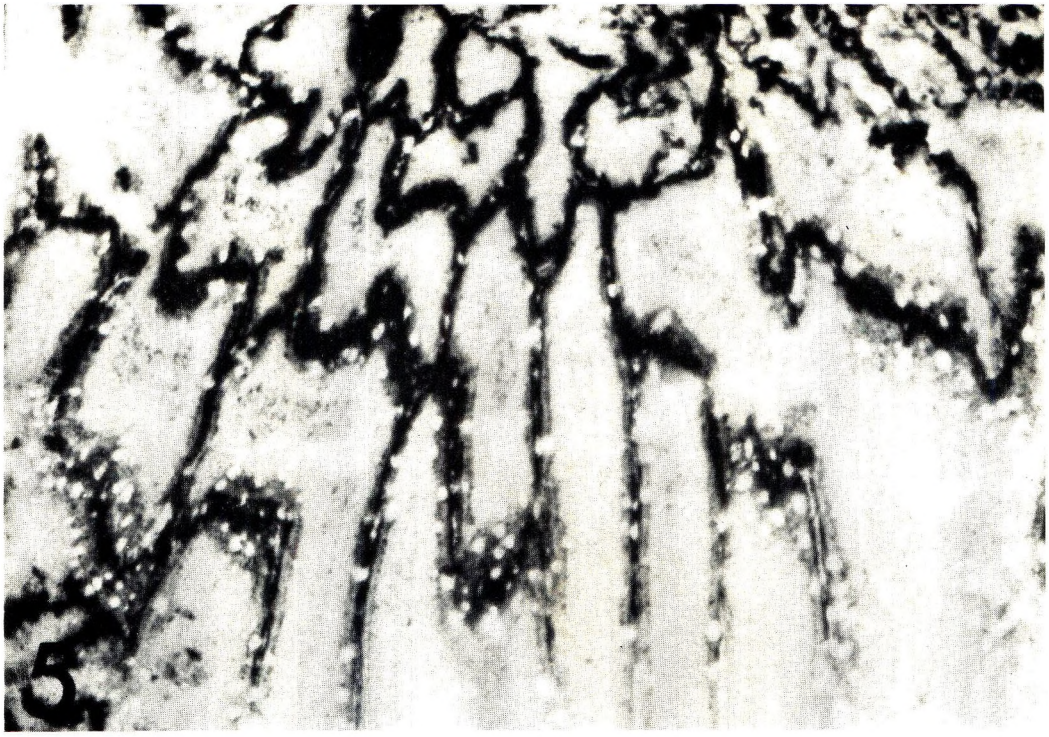

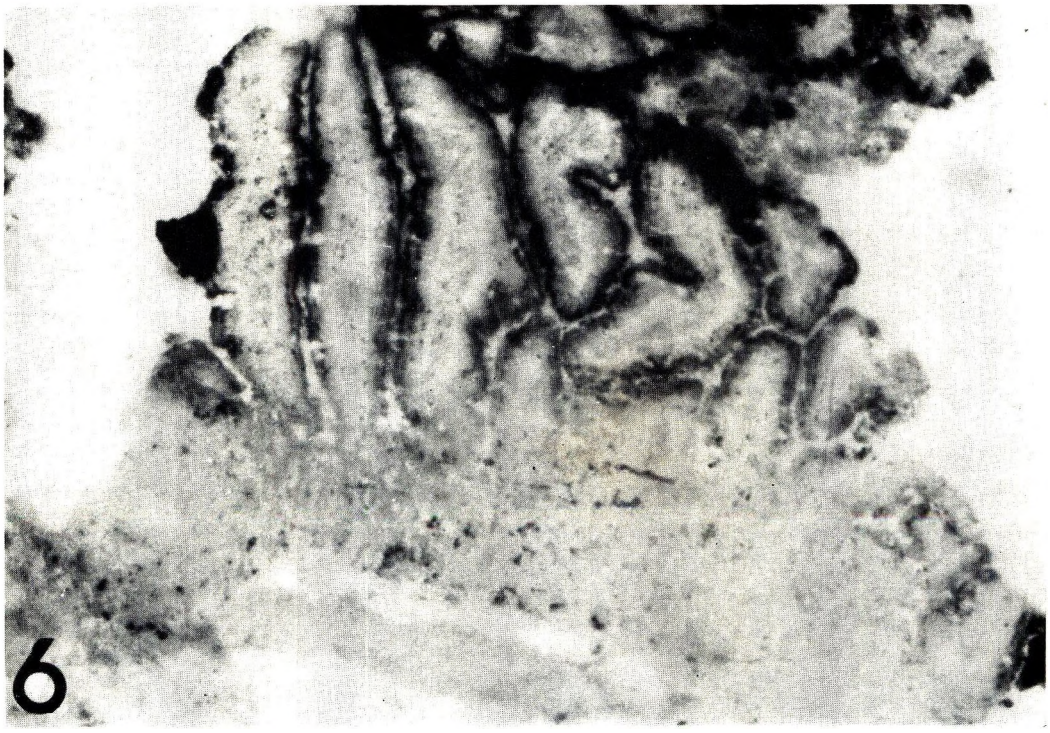

Figures 3 through 6 show the histochemical results for the enzymes acid phosphatase and succinic dehydrogenase. The activities of these enzymes in the absorptive cells showed a significant decrease in the jejunal tissue of protein-depleted rats at both the 5- and 10-week periods of study. Alkaline phosphatase, adenosine triphosphatase, and glucose-6 phosphatase were not observed to change and therefore these results are not shown.

Figures 7 through 10 show typical electron micrographs of the jejunum obtained from control and protein-depleted rats. The ultrastructure of the absorptive cell was well preserved in the protein-depleted rats. The microvilli and their membranous structures were found to be intact (figs. 8, 9), together with the presence of a dense ribosome population and associated endoplasmic reticulum (fig. 10). Generally, the mitochondria appeared normal with intact cristae and there was no evidence of mitochondrial swelling. The lysosomal population was sparse (fig. 8) and a normal Golgi apparatus was observed (fig. 10). Comparable normal fine structure was evident for the villus and crypt cells in both the protein-depleted and normal, well-nourished rats.

DISCUSSION

The present histochemical study demonstrates decreased acid phosphatase and succinic dehydrogenase activities in the absorptive cells of the jejunal villi of rats after 10 weeks of protein depletion. Since the substrates and incubation times were uniform for both control and protein-depleted rats, the differences in the enzymatic staining for these enzymes are assumed to reflect an actual difference in the concentration of these enzymes. Studies by Stenram (24) suggest that acid phosphatase activity of the jejunal mucosa is not altered by feeding rats isocaloric high protein, fat, or carbohydrate diets for a 3-month period. His studies did not include experiments with low protein diets and, therefore, these observations cannot strictly be compared with those obtained in the present study. Alkaline phosphatase, adenosine triphosphatase, and glucose-6 phosphatase activities were apparently unaffected in the present experiment, and ultrastructure of the absorptive cells of the villi was well preserved.

It would be difficult to correlate the histochemical changes in acid phosphatase with the present ultrastructural observations because a quantitative biochemical estimation of lysosomal activity in the jejunal villi was not carried out. The functional significance of diminished acid phosphatase activity is not entirely clear, but it has been reported that this enzyme is related to the development and actual activity of the pinocytic and reverse pinocytic processes which may participate in an intracellular digestive function. This implies that the enzyme may be involved in a defensive function rather than a nourishing one (25). It is interesting to speculate that a decrease in acid phosphatase activity may impair the intracellular defensive mechanism and increase the risk for villus damage due to a variety of toxins passing through the lumen of the small bowel.

The decrease in the mitochondrial enzyme, succinic dehydrogenase, was not paralleled by a change in the fine structure of the mitochondrion. Hill et al. (12) also report that the mitochondria were normal in the absorptive cells of the rat jejunal mucosa after 20 days of protein-free feeding. An alteration in the succinic dehydrogenase activity has been observed in clinical malabsorption before obvious morphological changes of the jejunal mucosa were noted by light microscopy (26), and this confirms earlier findings reported in experimental animals (27, 28). The present study suggests that the succinic-dehydrogenase activity is reduced even before any clear abnormality of the mitochondria is noted by the electron microscopic studies. The significance of the diminished succinic dehydrogenase requires further consideration, but it presumably reflects a depression in the intensity of oxidative metabolism in the jejunal cell.

The lack of a change in glucose-6 phosphatase activity correlated with the maintenance of the rough endoplasmic reticulum as revealed by electron micrographic study. This also correlates with earlier biochemical studies which show that the protein and RNA content of the intestinal

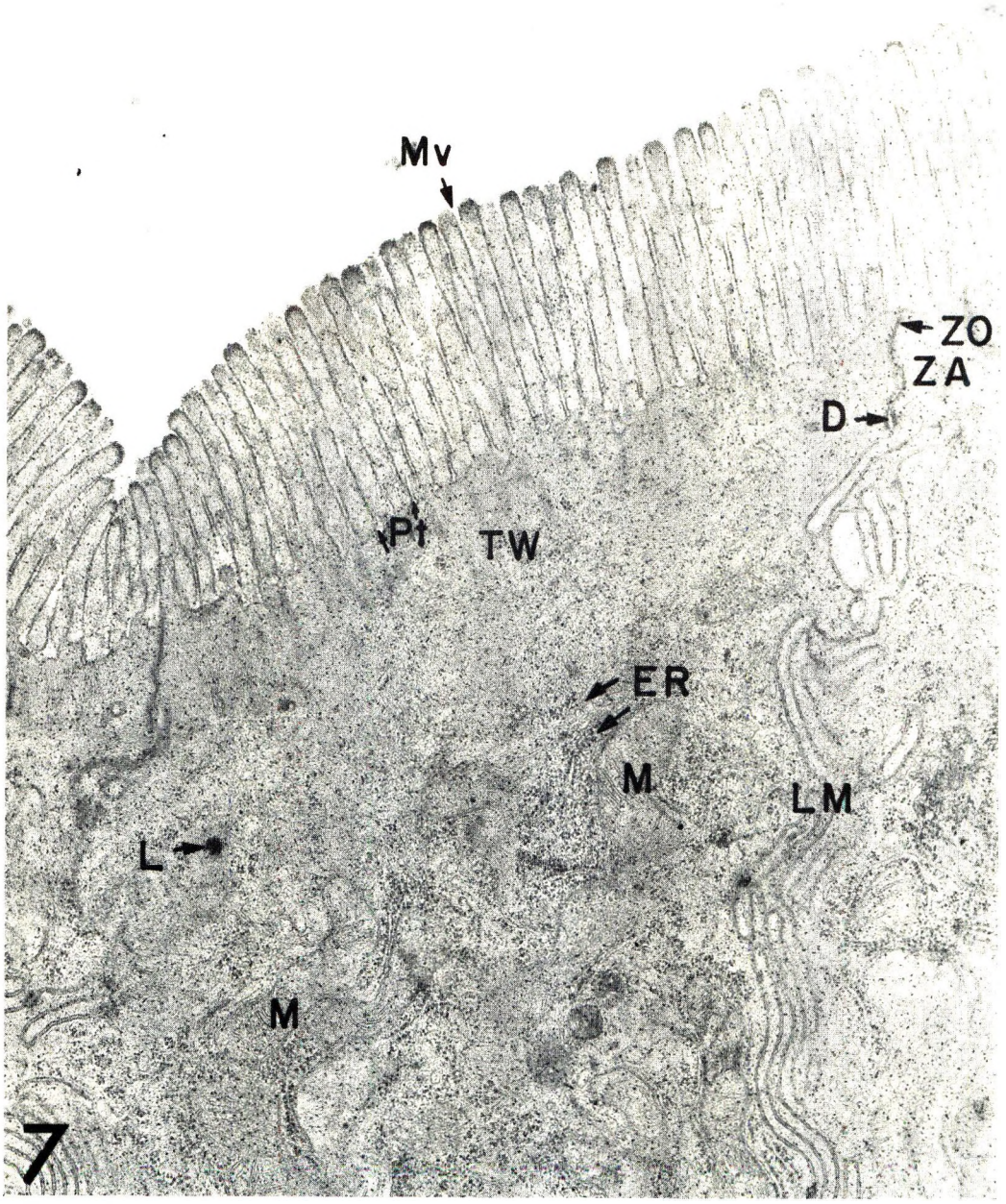

Fig. 7 Electron micrograph of the apical portion of the columnar absorptive cells from the jejunum of a control rat. Mv, microvilli; TW, terminal web; Pt, pits formed by the plasma membrane and penetrating into the terminal web; LM, lateral membrane with its interdigitating folds; ZO, zonula occludens; ZA, zonula adhaerens; and D, desmasome, the components of junctional complex at the lateral surfaces of cells; M, mitochondria with numerous cristae; L, lysosome; ER, endoplasmic reticulum. × 21,720.

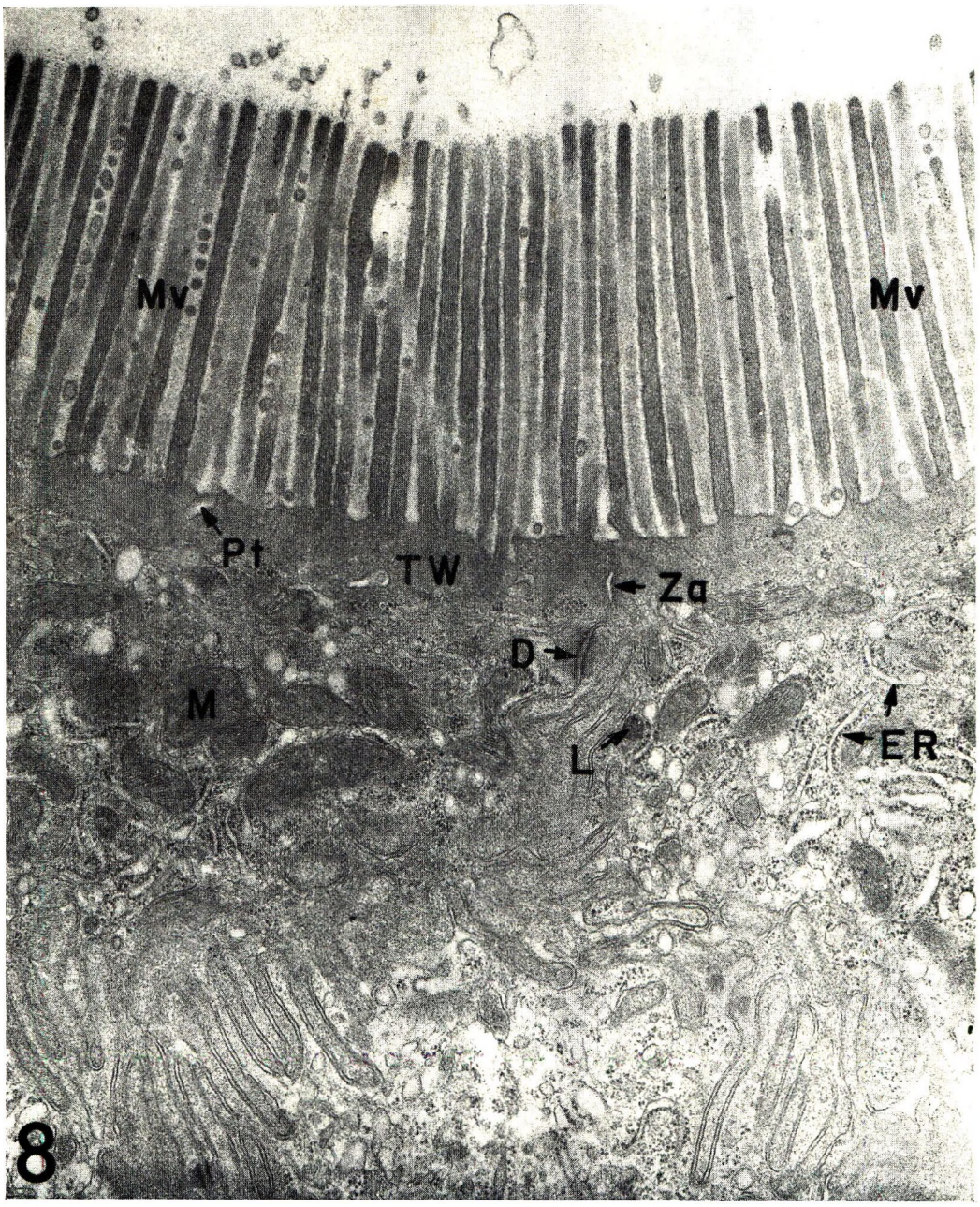

Fig. 8 Electron micrograph of the apical portion of the columnar absorptive cells from the jejunum of a rat fed the low protein diet for 10 weeks. Mv, well preserved microvilli; TW, terminal web; Pt, pit; Za, zonula adhaerens; D, desmasome; M, mitochondria with normal cristae; ER, intact abundant endoplasmic reticulum; L, lysosome. Ultrastructure of the cell is well preserved. × 21,720.

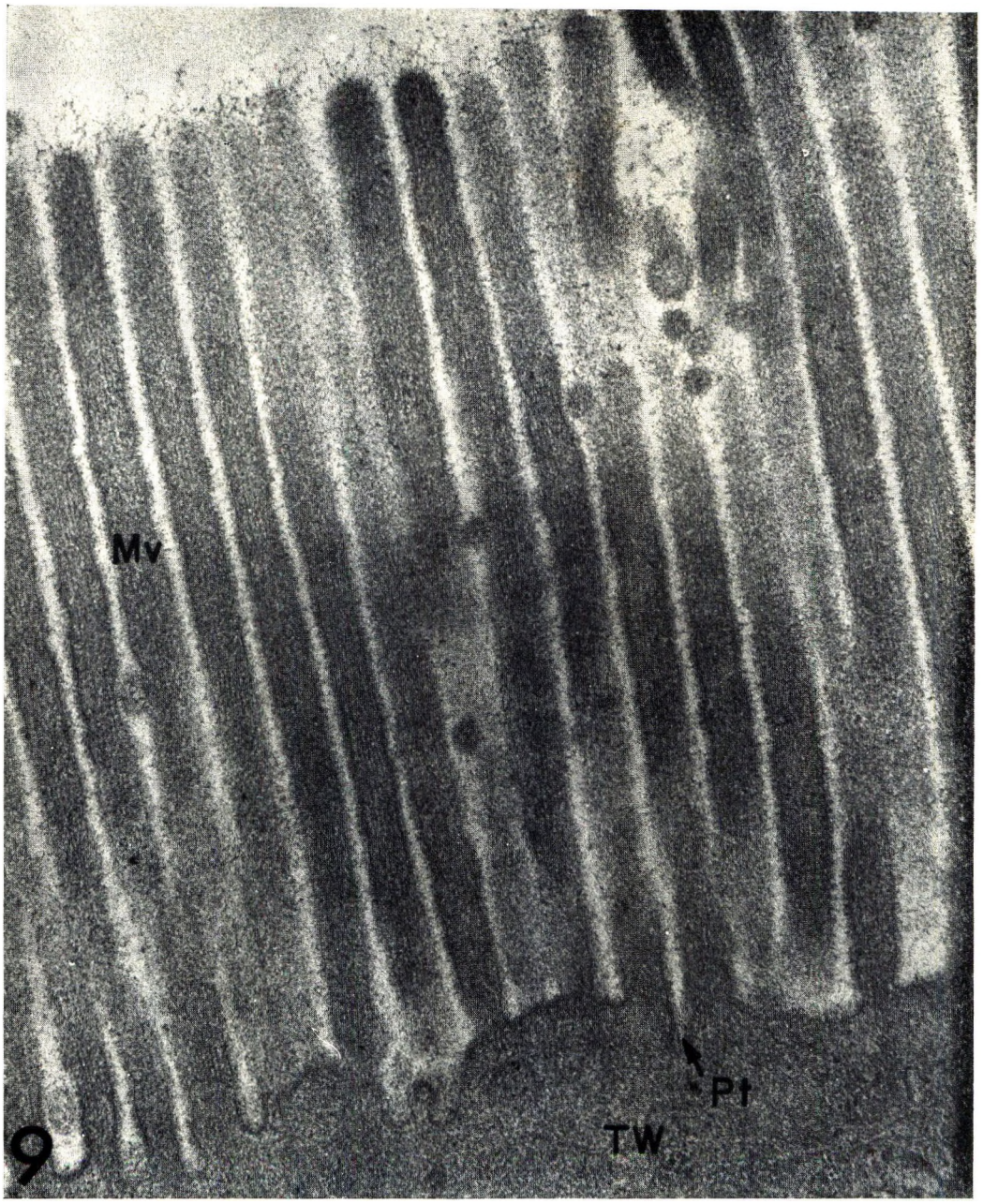

Fig. 9 Electron micrograph of microvilli of the columnar absorptive cell from the jejunum of a protein-depleted rat at the end of the experiment. Mv, microvilli with well preserved fine filaments which constitute the cores of these projections and extend into the terminal web (TW); Pt, pit. Surface coat of polysaccharide material covering the microvilli and projecting into the lumen of the bowel is intact. × 63,610.

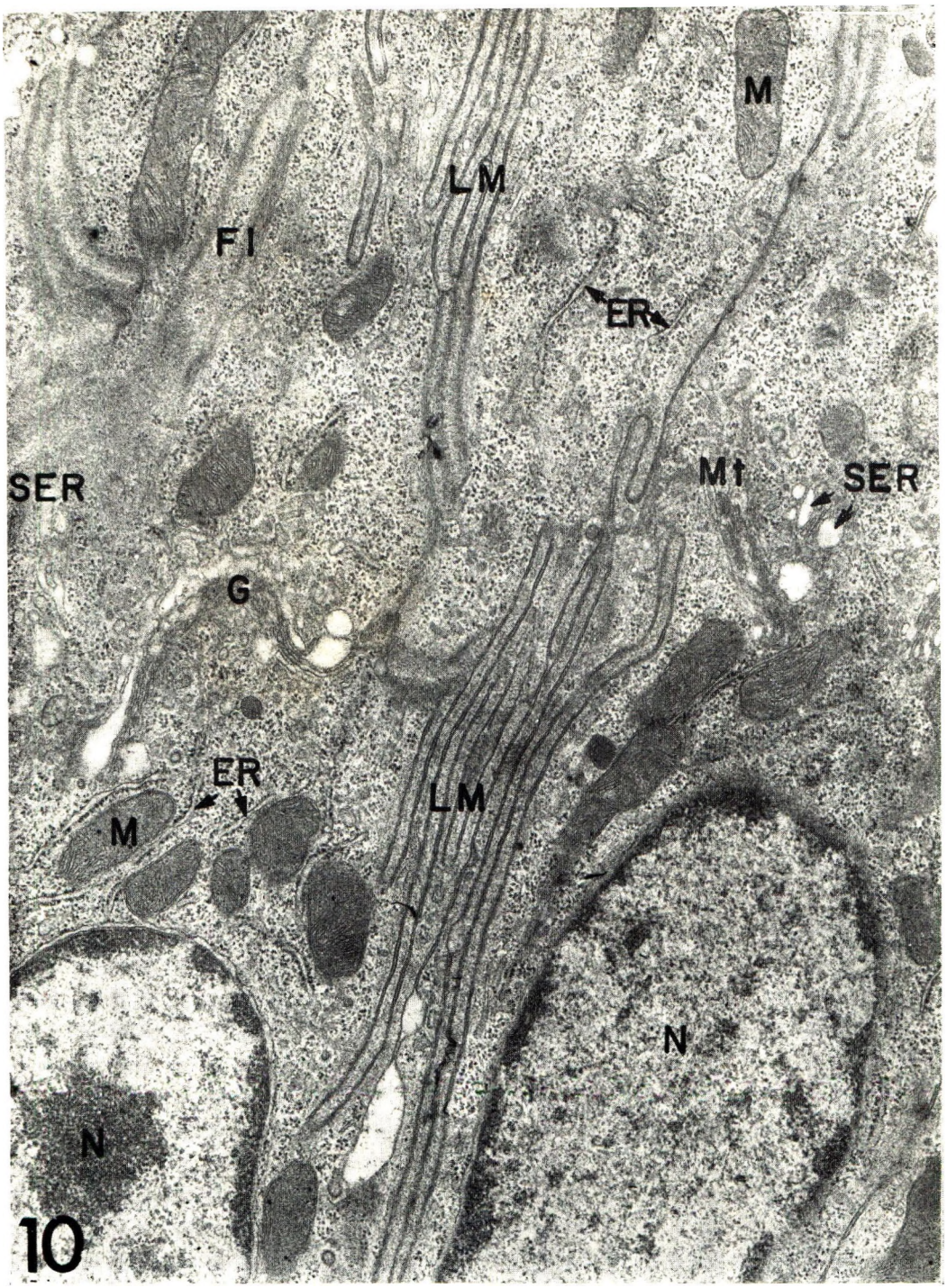

Fig. 10 Electron micrograph of the supranuclear portion of the columnar absorptive cell from the jejunum of a protein-depleted rat at the end of the experiment. All the intracellular organelle and membranous structures are well preserved. M, mitochondria with intact cristae; Fl, filaments; Mt, microtubules; LM, membrane with interdigitating folds at the lateral surfaces of cells; ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum free of ribosomes; G, Golgi complex; N, nucleus. × 21,720.

epithelial cell is not altered in protein-depleted rats (29).

The brushborder enzymes, alkaline phosphatase and adenosine triphosphatase, were unaffected by protein depletion and microvilli were well preserved. There do not appear to be detailed studies on the microvilli in protein depletion, but Hill et al. (12) have reported that a 20-day protein-free period did not alter the microvilli of the epithelial cells in the rat jejunum. Comparable studies are not available on the effects of protein depletion on brushborder enzymes. Stenram (24) reports no change in the alkaline phosphatase activity of small bowel mucosa in rats fed on isocaloric high carbohydrate, fat, or protein diets for a 3-month period.

The significance of microvilli for the absorption of nutrients is well established (30), and the role of brushborder enzyme alkaline phosphatase in the absorption of fat has been suggested (31). The absence of changes in the ultrastructure and enzyme histochemistry of microvilli after 10 weeks of protein depletion in rats may suggest that a dietary protein deficiency alone does not account for the severe morphological changes of the intestinal villi and associated malabsorption of nutrients observed in clinical protein-calorie malnutrition. The decrease in total absorptive area due to shortening of the villi would lead to some degree of malabsorption syndrome, but it appears likely that protein malnutrition acts as a predisposing factor for infections which may, therefore, be responsible for severe structural and functional changes of the small bowel as noted in children and adults with protein-calorie malnutrition. On the basis of their findings on disaccharidase activities in the jejunal mucosa of rats fed a low protein or protein-free diet, Solimano et al. (11) have offered a similar hypothesis for the pathogenesis of disaccharide intolerance in malnourished children.

ACKNOWLEDGMENTS

The authors thank Dr. Nevin S. Scrimshaw for his encouragement and Dr. L. Heimer, Mr. G. Villaire and Miss Nora Kula for their help during the conduct of the study.

LITERATURE CITED

1. Trowell, H. C., J. N. P. Davies and R. F. A. Dean 1954 Kwashiorkor, ed. 1. Edward Arnold, London, p. 149.
2. Stanfield, J. P., M. S. R. Hutt and R. Tunnelifle 1965 Intestinal biopsy in kwashiorkor. Lancet, 2: 519.
3. Mayoral, L. G., K. Tripathy, F. T. Garcia and J. Ghitis 1967 Protein malnutrition: induced malabsorption versus tropical sprue. Gastroenterology, 53: 507.
4. Tandon, B. N., M. L. Magotra, A. K. Saraya and V. Ramalingaswami 1968 Small intestine in protein malnutrition. Amer. J. Clin. Nutr., 21: 813.
5. Deo, M. G., and V. Ramalingaswami 1964 Absorption of Co^{58} labeled cyancobalamin in protein deficiency experimental study of Rhesus monkey. Gastroenterology, 46: 167.
6. Takano, J. 1964 Intestinal changes in protein-deficient rats. Exp. Mol. Pathol., 3: 224.
7. Platt, B. S., C. R. C. Heard and R. J. C. Steward 1964 The effects of protein-calorie deficiency on the gastrointestinal tract. In: The Role of the Gastrointestinal Tract in Protein Metabolism, ed. H. N. Munro. Blackwell Scientific Publications, Oxford, p. 227.
8. Svoboda, D., H. Grady and J. Higginson 1966 The effects of chronic protein deficiency in rats. II. Biochemical and ultrastructure changes. Lab. Invest., 15: 731.
9. Deo, M. G., and V. Ramalingaswami 1967 Reaction of small intestine to induced protein malnutrition in Rhesus monkey. A study of cell population kinetics in the jejunum. Gastroenterology, 49: 150.
10. Hopper, A. F., R. W. Wannemacher, Jr. and P. A. McGovern 1968 Cell population changes in the intestinal epithelium of the rat following starvation and protein depletion. Proc. Soc. Exp. Biol. Med., 128: 695.
11. Solimano, G., E. A. Burgess and B. Levin 1967 Protein-calorie malnutrition: Effect of deficient diets on enzyme levels of jejunal mucosa of rats. Brit. J. Nutr., 21: 55.
12. Hill, R. B., Jr., J. Prosper, J. S. Hirschfield and F. Kern, Jr. 1968 Protein starvation and the small intestine. I. The growth and morphology of the small intestine in weanling rats. Exp. Mol. Pathol., 8: 66.
13. Prosper, J., R. L. Murray and F. Kern, Jr. 1968 Protein starvation and the small intestine. II. Disaccharidase activities. Gastroenterology, 55: 223.
14. Edozien, J. C. 1968 Experimental kwashiorkor and marasmus. Nature, 220: 917.
15. Annino, J. S. 1964 Clinical Chemistry. Little, Brown and Company, Boston, Mass., p. 184.
16. Burstone, M. S. 1958 Histochemical comparison of naphthol-AS-phosphatases for the demonstration of phosphatases. J. Nat. Cancer Inst., 20: 601.
17. Wachstein, M., and E. Meisel 1957 Histochemistry of hepatic phosphatases at a physiologic pH. Amer. J. Clin. Pathol., 27: 13.

18. Barka, T. and P. J. Anderson 1965 Histochemistry Theory-Practice and Bibliography. Harper & Row, New York, p. 238.
19. Nachlas, M. M., K. C. Tsan, E. DeSanfa, C. S. Chang and A. M. Seligman 1957 Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrafole. J. Histochem. Cytochem., *5:* 422.
20. Wachstein, M., and E. Meisel 1956 On the histochemical demonstration of glucose-6 phosphatase. J. Histochem. Cytochem., *4:* 592.
21. Pearse, A. G. E., and E. O. Riecken 1967 Histology and cytochemistry of the cells of the small intestine in relation to absorption. Brit. Med. Bull., 23: 217.
22. Sabatini, D. D., K. G. Bensch and R. J. Barrnett 1962 New fixatives for cytological and cytochemical studies. In: Electron Microscopy. V. International Congress for Electron Microscopy, ed. S. S. Bresse, Jr. Academic Press, New York, p. L–3.
23. Marinozzi, V., and A. Gautier 1962 Fixations et colorations. Etude des affinités des composants nucléoprotéiniques pour l'hydroxyde plomb et l'acétate d'uranyl. J. Ultra-
24. Stenram, V. 1954 The effect of high carbohydrate, fat, or protein diet and of starvastruct. Res., *7:* 436.
tion on the histochemically determined phosphatases in the epithelium of small intestine in rat. Acta Anat., 20: 101.
25. Barka, T. 1963 Fat absorption and acid phosphatase activity in the intestinal epithelium of mice. J. Amer. Med. Ass., *183:* 761.
26. Myren, J., E. Gjone and S. Skrede 1965 Succinic dehydrogenase activity and histochemical changes of the small intestine mucosa in patients with gastrointestinal disease. Acta Pathol. Microbiol. Scand., *64:* 161.
27. Luketic, G. C., J. Myren, G. Sachs and B. I. Hirschowitz 1964 The effect of therapeutic doses of colchicine on oxidative enzymes in the intestine. Nature, 202: 608.
28. Myren, J., G. C. Luketic, G. Sachs and B. I. Hirschowitz 1964 The effect of colchine on absorption and dehydrogenase activity of small intestine. American Congress of Gastroenterology Research, Dallas, Texas.
29. Munro, H. N., and D. M. Goldberg 1964 The effect of protein intake on the protein and nucleic acid metabolism of the intestinal mucosa cell. In: The Role of the Gastrointestinal Tract in Protein Metabolism, ed., H. N. Munro. Blackwell Scientific Publications, Oxford, p. 189.
30. Schiner, M. 1967 Ultrastructure of jejunal surface epithelium in untreated idiopathic steatorrhea. Brit. Med. Bull., 23: 223.
31. Prezelecka, A., G. Ejsmont, M. Sarzala and M. Taracha 1962 Alkaline phosphatase activity and synthesis of intestinal phospholipids. J. Histochem. Cytochem., *10:* 596.
32. Rogers, Q. R., and A. E. Harper 1965 Amino acid diets and maximal growth in the rat. J. Nutr., *87:* 267.

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