Effects of Maternal Protein Restriction on the Kidney of the Newborn Young of Rats[1]

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ABSTRACT The effect of maternal protein restriction on the kidney of the young rat was studied. Kidneys were taken from newborn young of rats fed semipurified diets containing 24% or 6% unsupplemented casein as the sole source of protein throughout pregnancy. There are definite morphological and histochemical differences between kidneys from the animals in the control and protein-deficient groups. Kidneys from the protein-restricted animals have fewer and less well-differentiated glomeruli, a greater proportion of connective tissue, and relatively fewer collecting tubules. PAS-stained sections indicate that proximal convoluted tubules are shorter and have fewer convolutions. Acid and alkaline phosphatase activities were reduced in kidneys of protein-deficient young, but no differences were found in the activities of ATPase, nonspecific esterase, or leucine aminopeptidase. Some changes also resemble those reported in kidneys of protein-deficient weanling rats. It is suggested that maternal protein restriction results in both immaturity in development in the kidneys of the young and in pathological changes symptomatic of protein deficiency.

It has been demonstrated previously that maternal protein restriction during pregnancy results in decreased birth weight in the young (1). The liver and kidneys of these young are smaller in proportion to total body weight than in the controls, indicating that these organs may be the most severely affected. The specific nature of the effects on these organs, however, has not been investigated.

It may be speculated that the condition of the kidneys of the young of protein-deficient pregnant rats might resemble those observed in weanling rats fed protein-calorie deficient diets (2). Alternatively, it has been suggested that organs which are disproportionately small in young animals may be immature (3, 4). The kidneys of the young of protein-deficient dams might, therefore, be immature but not show any pathological changes suggestive of protein deficiency.

The present work was undertaken to clarify the specific effects of maternal protein deficiency on the kidneys of newborn young.

MATERIALS AND METHODS

Virgin, female rats of the Sprague-Dawley strain were divided into 3 groups and mated when at a body weight between 180 and 200 g. Pregnancy was considered to have begun when vaginal plugs and sperm were found. This was designated as day zero of pregnancy.

At this time, animals in the control group were fed ad libitum a diet containing dextrose,[2] 62%; casein,[3] 24%; corn oil, 8%, and salt mix,[4] 6%. The diet of the experimental group contained 6% unsupplemented casein and an additional 18% carbohydrate. Each animal in the third, or pair-fed, group received an amount of control diet equal to the ad libitum intake of a female in the protein-restricted group which was of equal weight on day zero of pregnancy. Each animal in each group received a vitamin supplement mixture[5] 3 times weekly. These diets were fed to the animals beginning on day zero and were continued throughout pregnancy.

Kidneys were removed from newborn young within 8 hours after birth. Animals that appeared moribund were discarded. One kidney from each pup was fixed in

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

[3] Nutritional Biochemicals Corporation, Cleveland.

[4] Salt content of diet: (g/kg) $CaCO_3$, 18.0; K_2HPO_4, 19.5; $CaHPO_4$, 3.6; NaCl, 10.08; $FeSO_4 \cdot 7H_2O$, 1.5; $MgSO_4 \cdot H_2O$, 0.0075; KI, 0.015; $ZnCO_3$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.018; and $MnSO_4 \cdot H_2O$, 0.138.

[5] Calculated on a per day basis, each pregnant or lactating animal received: (in milligrams) choline, 20.0; inositol, 10.0; ascorbic acid, 2.0; Ca pantothenate, 1.0; (in micrograms) *p*-aminobenzoic acid, 200.0; pyridoxine, 600.0; nicotinic acid, 600.0; riboflavin, 200.0; thiamine, 600.0; menadione, 500.0; biotin, 5.0; folic acid, 12.0; vitamin B_{12}, 0.6; and vitamin A palmitate, 300 IU; vitamin D, 30 IU; and *dl*-α-tocopherol, 2.2 IU.

calcium formalin, dehydrated, cleared, and embedded in paraffin in the usual manner. Paraffin sections were cut at 6 μ and stained with hematoxylin and phloxine B (5), alcian blue-PAS (5), or toluidine blue (6).

The second kidney from each animal was fixed in cold calcium formalin for 48 hours and transferred to gum sucrose for 1 to 3 days. Cryostat sections, 10-μ thick, were stained for alkaline phosphatase (6), ATPase (7), acid phosphatase (7), nonspecific esterase (6), and leucine aminopeptidase (6).

Quantitation of morphological components by microscopic methods is admittedly difficult. To avoid errors of duplication, the number of mature and immature glomeruli were counted in a single hematoxylin and phloxine-stained section cut through the hilus at 6 μ. Immature glomeruli, in conformity with Potter's classification, included nephrogenic masses, vesiculated nephrogenic buds, S-shaped anlagen, primitive hemispheric glomeruli, and those having proliferating capillary tufts and cuboidal parietal epithelium (8). Each section was taken from one kidney from each of 10 animals from 8 litters in each group. In addition, the proportion of cross sections of proximal convoluted tubules in relation to numbers of glomeruli were counted in the PAS-stained sections.

RESULTS

The results indicate definite morphological and histochemical differences between the kidneys of the control and protein-deficient newborn, but no observable differences between kidneys from the control and from the pair-fed group. Figure 1 shows, at identical magnification, paraffin sections of kidneys from the control and protein-restricted pups, stained with hematoxylin and phloxine B. The size difference is very obvious. Kidneys from the deficient group have a greater proportion of mesenchymal-like connective tissue and relatively fewer collecting tubules in the medullary rays. Examination of these tissues with in-

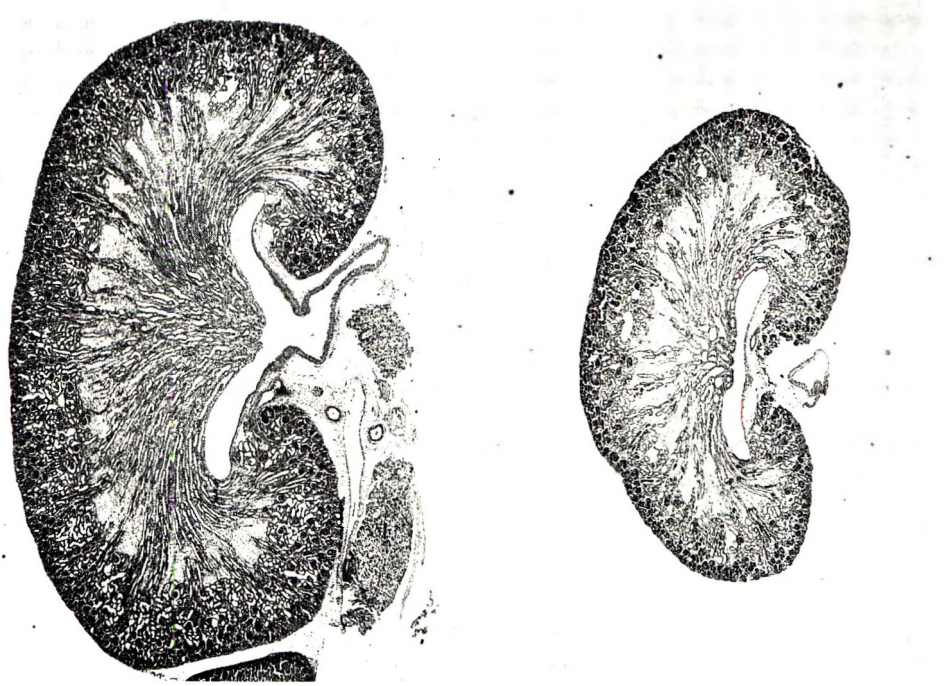

Fig. 1 Coronal sections through hilus of kidneys of newborn young of dams fed 24% casein diet (left) and 6% casein diet (right). Hematoxylin & phloxine B. × 8.

creased magnification shows that the neogenic zone is wider in proportion to the total width of the cortex and, consequently, the number of immature glomeruli in proportion to the total appears to be increased. The formed glomeruli are fewer in number and are less well differentiated. The capillary tuft appears more compact and the parietal epithelium in many of these glomeruli is cuboidal. Pre-glomerular structures are present but have undergone minimal differentiation. Some S-shaped anlagen are present in both controls and deficients, but are fewer in the controls. Table 1 gives numbers of mature and immature glomeruli and of total glomeruli in sections through the hilus.

TABLE 1

Effect of maternal protein restriction in the rat on glomerular development in the young

	Diet	
	24% casein	6% casein
Total glomeruli hilar section	147.4 ± 21.04 [1]	121.6 ± 21.21 [2]
Immature glomeruli, %	30.9 ± 6.75	46.3 ± 6.8 [3]

[1] Mean ± one SD.
[2] $P = < 0.05$.
[3] $P = < 0.001$.

High-power photomicrographs of PAS-stained sections (fig. 2) show a mature glomerulus from an animal in each group and the cross sections of proximal convoluted tubules found in the immediate vicinity. Fewer cross sections are associated with the glomerulus in the kidney from the 6% casein group on the right, compared with the number of cross sections in the control tissue on the left.

In addition, it was observed in the low protein tissue that the cytoplasm of the proximal convoluted tubules did not stain with PAS. The cells appear enlarged with rounded, pale-staining nuclei. The basement membrane is intact and well-stained. The luminal surfaces of the proximal convoluted tubules show a decreased staining reaction to Schiff's, and no discrete brush border is identifiable.

Sections stained with toluidine blue show no differences in metachromasia between the 2 groups. Connective tissue is metachromatic in both groups.

Acid phosphatase activity, indicated by the darkened areas in the sections in figure 3, was noted in the more mature proximal convoluted tubules in sections taken from kidneys of the control group, but was ab-

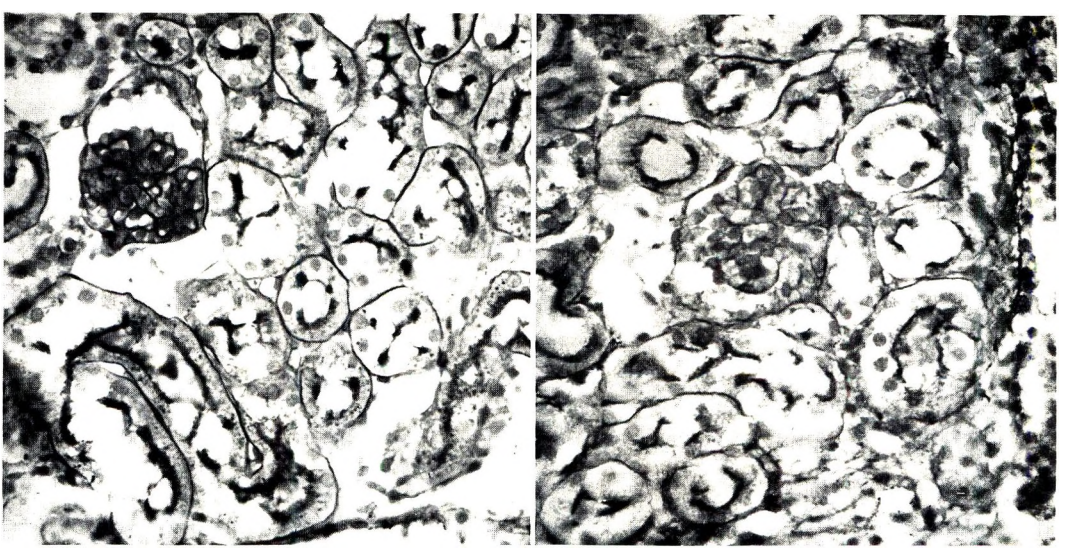

Fig. 2 Cortex of kidneys of newborn young dams fed 24% casein diet (left) and 6% casein diet (right). In section at right note fewer proximal tubular cross sections with decreased staining of luminal surfaces and differences in cell size. Alcian blue-PAS. × 200.

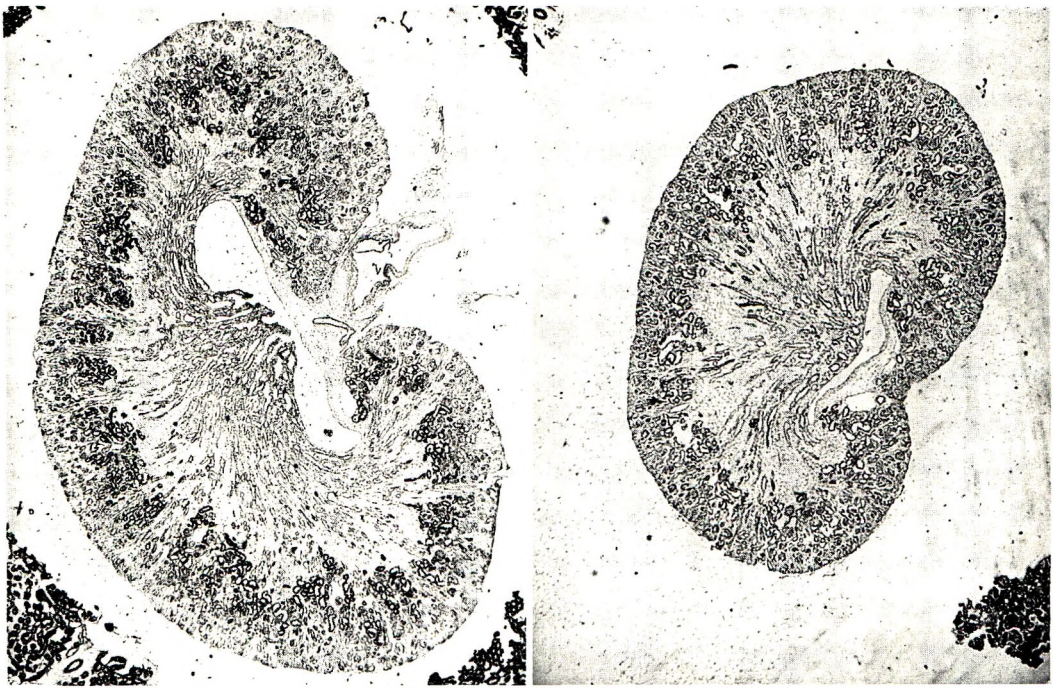

Fig. 3 Acid phosphatase activity in kidneys of newborn young of dams fed 24% casein diet (left) and 6% casein diet (right). × 8.

sent from the sections of kidneys from the 6% casein group. The tissue fragments appearing in the corners of these illustrations are normal adult kidney which was used as both an embedding device and as a positive control for the staining procedure.

Sections taken from kidneys of animals in the control group and stained for alkaline phosphatase also showed evidence of enzyme activity in the more mature proximal convoluted tubules. Sections taken from kidneys of pups in the deficient group had considerably less activity or, in some cases, none.

No differences in the development of activity of esterase, ATPase, or leucine aminopeptidase were found.

DISCUSSION

Kidneys of pigs, dogs, and rats fed protein-calorie deficient diets have been described as showing loss of cell cytoplasm in the proximal convoluted tubules, loops of Henle, and collecting tubules. Some cells of the proximal convoluted tubules showed cloudy swelling. The brush borders were less strongly PAS-positive than those of well-fed controls (2). Kidney sections obtained from the protein-restricted group in this study demonstrate some of the above changes. A great many of the differences present, however, are those which suggest immaturity since the kidneys of protein-deficient animals resemble fetal kidneys of normal animals.

These observations suggest that a major effect of intra-uterine protein malnutrition on the kidneys of the young may be a retardation in development. In the normal rat fetus at 18 days' gestation, the kidney consists mostly of stellate mesenchyme with a few developing glomeruli, a few collecting tubules, but no identifiable proximal tubules. Proximal tubules are found at 19 days. At 20 days, they are more numerous, as are the developing glomeruli and collecting tubules. At birth, there is normally little remaining mesenchymal tissue (9).

Renal immaturity in the protein-deficient young in this study is indicated by increased quantities of mesenchymal-like connective tissue, fewer identifiable glomeruli, and a larger proportion of immature glomeruli.

The kidney of the rat is not fully formed at birth, but resembles morphologically the embryonic kidney of species, such as man, in whom nephron formation is complete at birth (10). Potter (8) describes the sequence of events in the developing human glomerulus, including the formation of S-shaped anlagen, the later flattening of epithelial cells of Bowman's capsule, and still later, lobulation of the glomerulus. The presence of greater numbers of these more immature forms in the wider neogenic zone in kidneys of the protein-deficient young is interpreted as additional evidence of immaturity.

There is evidence that the large amount of mesenchymal-like connective tissue is not abnormal when metachromasia is used as the criterion of chemical composition. It is suggested, instead, that this tissue is simply less differentiated tissue such as that in younger fetuses.

The presence of fewer proximal tubules or shorter tubules in young fetuses results in decreased numbers of tubular cross sections in kidney tissue sections. The lesser number of tubular cross sections found in the kidneys taken from newborn protein-deficient young in this study is, therefore, further evidence of renal immaturity.

The evidence of decreased alkaline and acid phosphatase activity in these young may also be considered to be indicative of retarded development since enzymogenesis is considered to be an aspect of differentiation (11, 12). In the kidney, as well as in other organs, the activity of all enzymes does not necessarily increase constantly as the animal develops (13–16), but may decrease at certain periods. In the cases of alkaline and acid phosphatases, however, histochemical evidence indicates activity in increasingly larger areas of the rat kidney as the animal matures prenatally (17). Therefore, a decrease in or absence of activity of these enzymes in the neonatal protein-deficient rat might also be considered to indicate immaturity.

In view of the lack of knowledge of functions of alkaline and acid phosphatases in the kidney, the specific effects of their depressed activity are unknown. Phosphatase activity has long been considered a sensitive indicator of the functional capacity of the kidney (18). Alkaline phosphatase has been shown to be limited to the brush border of the proximal convoluted tubules in human (19) and rat (20) kidneys. Its activity is first exhibited along with the development of the brush border and has been considered to indicate the progress of differentiation of the proximal convoluted tubules (21). It has been suggested that this enzyme functions in tubular absorption phenomena (22, 23). Tubular reabsorption may therefore be compromised in the protein-deficient young.

The absence of activity of acid phosphatase may indicate decreased lytic activity. This enzyme has been used as an indicator of the presence of lysosomes in rat liver (24) and rat kidney (25, 26) and has been associated with lytic activity in the adult mouse (27). Presumably, general retardation of development would also affect the other enzymes studied. No explanation is available for the differences found between activities of alkaline and acid phosphatases, on the one hand, and of ATPase, esterase, and leucine aminopeptidase, on the other. It appears that the effects of intra-uterine protein deficiency on the kidneys of the young are not simply the result of retardation of maturation processes, but that other mechanisms are also involved. Further investigation is needed.

The persistence of the reported changes poses another problem for investigation. Winick and Noble (28) have reported that recovery from growth failure caused by general malnutrition is less likely with earlier onset of the malnutrition. They report that in early growth failure there is depressed cell division.

If this relationship applies in the present situation, the protein-deficient young in this study should be permanently stunted. Presumably, cell division in the kidney might be compromised so that kidney structures may never reach normal size or normal development.

The consequences of these histological and histochemical differences in terms of kidney function are also unknown. It might be expected that both glomerular and tubular function would be reduced. The results of tests of kidney function, now in progress, should help to clarify this point.

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Iodine Uptake by Ova of Hens Given Excess Iodine and Effect upon Ova Development [1,2]

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ABSTRACT White Leghorn hens were dosed orally for 14 days with a solution of sodium iodide labeled with ^{131}I and iodine excretion into developing ova, eggs, whole body and selected tissues was determined. Daily dosage levels were 100 mg in a single dose, 500 mg in 2 administrations or 500 mg in a single dose. Iodine in the eggs of hens given 100 mg iodine daily increased linearly for 10 days and reached a plateau of approximately 3 mg/egg at that time. Concentration in the eggs from those given 500 mg increased rapidly to an average content of 7 mg/egg by 8 days at which time most hens ceased production. Ova continued to develop in hens not laying and many ova were found to be regressing. It is suggested that when a threshold amount of iodine reaches the ova, development ceases and regression takes place.

Several recent reports from this laboratory (1, 2) and earlier reports by others (3, 4) have described some of the effects of excess dietary iodine upon laying hens. Egg production ceased or was reduced depending upon the intake of iodine, but production resumed shortly after iodine feeding was stopped. Fertility of eggs was not affected but high embryonic mortality and delayed hatching were observed when eggs were incubated. The mechanism by which the excess iodine affects egg production and embryonic mortality is not understood. Preliminary studies [4] of thyroxine production in hens fed high levels of iodine have indicated that production of the hormone was not impaired.

The present study was conducted to determine the uptake of iodine by ova at different stages of maturation, the patterns of excretion into eggs and iodine content of eggs, whole body and selected tissues of hens dosed orally with high levels of iodine.

EXPERIMENTAL

Thirty-six White Leghorn hens, laying at a rate of 75% or more, were used in 3 trials. All were housed individually in wire laying cages and provided tap water and a practical layer diet ad libitum. The diet consisted of the following: (in percent) ground yellow corn, 67.7; soybean meal, 20.6; alfalfa meal, 3.0; ground limestone, 5.8; defluorinated phosphate, 1.96; iodized salt, 0.4; and microingredients, 0.5. The microingredients supplied per kg diet: vitamin A, 4,400 IU; vitamin D_3, 1,540 ICU; and (in milligrams) choline, 500; niacin, 13.2; riboflavin, 4.4; Ca pantothenate, 8.8; ethoxyquin, 12.5; $MnSO_4$, 220; and (in micrograms) vitamin B_{12}, 13.2. Iodine was administered as a solution of sodium iodide labeled with ^{131}I which had an initial specific activity of 0.1 μCi/mg or 0.024 μCi/mg of iodine. Solutions were administered with a small pipette inserted deep into the esophagus.

In the first trial, 100 mg iodine per hen were given in a single dose. Five hundred milligrams divided into 2 daily increments were given in the second trial. Birds in the third trial were also given 500 mg daily but the amount was administered in a single dose. The 100-mg daily amount was equivalent to 1000 ppm dietary iodine and 500 mg were equivalent to 5000 ppm in the diet, which had been shown previously to stop egg production after one week (2). The daily administration was carried out for 14 days in all trials and continued to 20 days for some hens in the last study.

Eggs were collected daily for the treatment period and thereafter until no activity could be detected in the egg. At the end of the last trial 6 hens which were laying

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[4] Unpublished data, N. A. Marcilese, 1966.

and 4 not laying were killed at 24 hours after the last dose. Iodine in the whole body, ovary and its contents, ova and in the gastrointestinal tract was determined.

Appropriate standards for each iodine solution administered were prepared in a volume and of geometry similar to the samples being counted. Radioactivity measurements were made with a 4-π scintillation detector and stable iodine was calculated from specific activity of the solutions administered. Total iodine in the hens before treatment was extremely low compared with that administered and was thus ignored in the calculation. Based upon estimates for man (5) of 0.1 to 0.4 mg/kg body weight, that amount present in hens would be several thousand times less than that administered.

RESULTS AND DISCUSSION

The iodine content of eggs increased almost linearly for 10 days and reached a plateau of approximately 3 mg per egg in hens dosed once daily with 100 mg of iodine (fig. 1). When administration was discontinued on day 14, the level in eggs decreased almost linearly with time and after 10 days no appreciable amount was detected.

The pattern of excretion into eggs from hens given 500 mg daily in 2 separate doses is shown in figure 2. At 8 days, egg production had ceased and the average

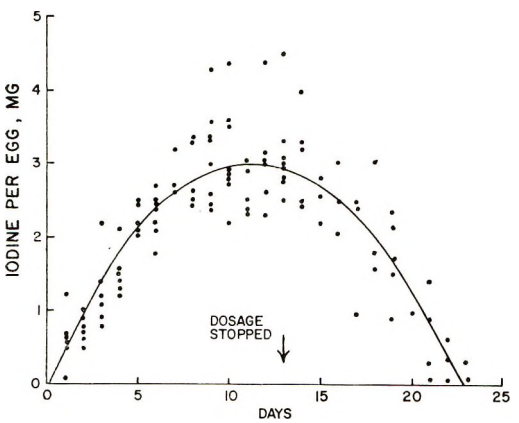

Fig. 1 Iodine content of eggs from hens during and following iodine treatment. Oral dosage: 100 mg daily for 14 days.

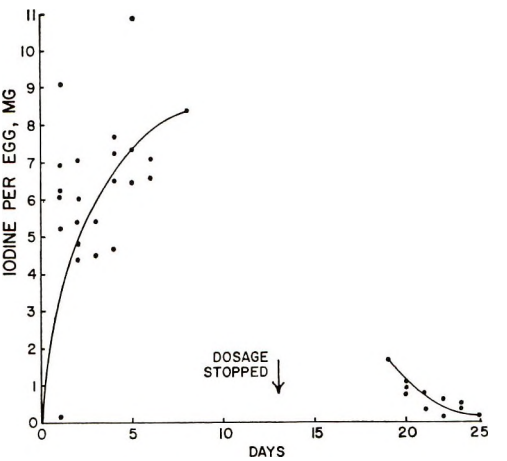

Fig. 2 Iodine content of eggs from hens given 500 mg iodine daily in 2 separate doses. No eggs produced after 8 days of administration but production resumed 5 days after iodine administration discontinued.

iodine content per egg at that time was approximately 7 mg. Six days after the iodine administration was discontinued, egg production resumed and eggs contained less than 2 mg of iodine.

The ability of the growing ova to concentrate iodine has been demonstrated by other workers (6–8). It was shown that with continuous administration of trace amounts of radioactive iodine, a plateau of about 10% of the daily dose was reached. Results of the present study show that ability to transfer iodine to ova remains effective when excessive quantities of the halogen are administered. The proportion transferred, however in this study was less than that transferred when trace amounts were given. The administration of 100 mg daily resulted in an average of 3 mg per egg which was 3% of the daily dose. When 500 mg were administered the amount per egg was approximately 7 mg which represented an even smaller proportion. Since egg production generally ceased when the concentration reached about 7 mg per egg, it may be considered that this represents a threshold.

When 16 hens were dosed once daily with 500 mg of iodine, 10 ceased production within 10 days and 6 continued to lay but at a lower rate. Iodine content of these

Fig. 3 Ova from hen given 500 mg iodine daily for 14 days and that had stopped laying. Note some developing ova and others undergoing regression.

eggs also reached a plateau of 7 to 8 mg and some even greater before egg production ceased. The average value for the last egg laid was 7.7 mg. The failure of 500 mg iodine, administered as a single daily dose, to completely suppress egg production may have resulted from decreased absorption. It may be expected that less absorption would take place from a single large dose than from the same amount extended over a longer period.

Iodine content in the whole body of hens laying and those that had stopped laying was similar and amounted to approximately 46 mg when that present in the gastrointestinal tract was subtracted (table 1).

TABLE 1

Iodine content of whole body, eggs and ova of hens given 500 mg iodine daily for 14 days [1]

	Laying [2]	Non-laying
Whole body, mg	50.1 ± 12.0 [3]	56.2 ± 17.0
Gastrointestinal tract, mg	3.0 ± 1.5	11.4 ± 4.8
Ovary and contents, mg	14.5 ± 3.7	6.8 ± 1.0
Egg in oviduct, mg	6.0 ± 0.7	—
Wt 5 largest yolks, g	32.5 ± 12.3	9.1 ± 1.5
Total I_2 in 5 largest yolks, mg	10.5 ± 4.0	4.7 ± 0.9
I_2 g yolk, mg	0.32 ± 0.04	0.52 ± 0.09

[1] Determinations made 24 hours after last dose.
[2] Six hens laying, 4 not laying.
[3] SD.

That present in the gastrointestinal tract of non-layers was considerably more than that of laying hens. Iodine in the ovary and contents of those laying was more than twice that of non-layers. The total weight and total iodine of the 5 largest yolks from laying hens was greater than that of non-layers, but on a unit-weight basis, the yolks from non-layers contained more iodine. Egg shells and albumen contained no appreciable amount of iodine.

The total number of ova in laying and non-laying hens was approximately the same. In all cases, the ova in those that had stopped laying were smaller and most were undergoing regression. Ova from a hen given 500 mg iodine and not laying are shown in figure 3. It was observed in a former study (2) that when egg production ceased as a result of excess iodine, ova continued to develop but ovulation did not take place. Several of the hens that continued to lay produced eggs with an amount of iodine equal to that at which others ceased production. The iodine in other follicles, however, was only about one-half that in the follicles of hens not laying. It is suggested that ova in the latter stages of maturation take up a larger amount of the available circulating iodine. These may still be ovulated if past a certain stage of development. The smaller or less developed ova, upon accumulation of the threshold amount of iodine appear to cease development and regression begins.

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Cellular Response with Increased Feeding in Pituitary Dwarf Mice[1]

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ABSTRACT Studies were made to determine whether an observed increased weight, with increased caloric intake, in neonatal pituitary dwarf mice is accompanied by acceleration in the rate of cell division. Decreasing nursing litter size in pituitary dwarf mice increased weight and protein content of the entire animal and of certain organs with the exception of brain. DNA content, however, was not affected. Longevity of these animals was at least 2 times that of dwarfs raised in standard-size litters. These data are interpreted as demonstrating that increasing the total caloric intake of pituitary dwarf mice will result in increased total weight which is manifested by increased individual cell size without an increase in cell number. It is concluded that anterior pituitary hormones, in vivo, may play a role in DNA synthesis independent of their role in protein synthesis.

Animal or organ growth may be serially followed by changes in total weight or more specifically by changes in cell number (total organ DNA), cell size (weight/DNA or protein/DNA), or both (1, 2). All three of these parameters are regulated, to some extent, by anterior pituitary hormones. Not only are weight and length reduced in the hypopituitary animal, but cell number and cell size are also reduced within individual organs (3).

A similar decrease in total organ DNA content and protein/DNA ratios occurs with severe malnutrition (4). Therefore, the lack of growth in hypopituitary animals could be due in part to a reduced caloric intake. The fact that these animals are nursing precludes the usual pair-feeding experiments. However, in normal animals, by decreasing the size of the nursing group, one can increase the quantity of food per animal (5), the growth rate of the animal (6), and the number of cells in all the organs except brain (7).

The purpose of the present experiments was to determine whether "overfeeding" animals completely devoid of anterior pituitary glands, in this manner, would result in an acceleration in growth and an increase in the rate of cell division.

MATERIALS AND METHODS

Pituitary dwarf mice of the Snell Smith strain (8, 9) were reared from birth in groups of 1 to 3 animals. Normal control animals of the same strain were reared in the same way. These "overfed" animals were compared with both dwarf and normal animals reared in the usual groups of 8 to 10 pups.

Animals were killed at weaning (21 days). Total animals and various organs (brain, heart, liver, and kidney) were weighed and analyzed for DNA and total protein by methods identical to those described previously (2). Other animals were weaned to stock laboratory ration and their growth was serially followed.

RESULTS

Weight. Table 1 demonstrates that the "overfed" control animal is heavier than its normal littermate. The hypopituitary animal is also considerably heavier at the time of weaning if reared in a group of 1 to 3 animals. Hence increasing total caloric intake increases weight in both control and dwarf animals.

Heart, kidney, and liver were significantly heavier in both "overfed" dwarfs and normals. Brain, however, showed no significant change in weight in either normal or dwarf animals regardless of litter size.

Total protein. The changes in total protein parallel those for weight in the whole animal in both dwarfs and controls (table

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TABLE 1

Organ weight at weaning in normal and dwarf mice [1]

	8-10 animals/litter	1-3 animals/litter	8-10 animals/litter	1-3 animals/litter
	Normal control	"Overnourished" control	Normal dwarf	"Overnourished" dwarf
	g	*g*	*g*	*g*
Brain	0.42	0.42	0.38	0.38
Heart	0.085	0.115	0.03	0.06
Kidney	0.186	0.24	0.069	0.110
Liver	0.775	0.90	0.176	0.30
Whole animal	12.0	16.0	6.0	10.0

[1] Each value represents the average for 5 separate animals with a variation of less than 7% for any individual animal within a particular group. There is no overlap between groups except in the brain. All other organs show a statistically significant difference between each of the 4 groups.

2). In the "overfed" control the increase in protein is proportional to the increase in weight. However, in the "overfed" dwarf the increase in protein is proportionally only about one-half the increase in weight. This is probably explained by increased deposition of adipose tissue which is visible in the dwarf. However, in heart, kidney, and liver the increased weight is accompanied by a proportional increase in total organ protein in both "overfed" dwarfs and controls. This would indicate an increase in protoplasmic mass rather than an abnormal deposition of fat within these organs. Moreover, histological examination appeared normal, again indicating no fatty deposition. As with weight, no change in protein content of brain was induced by increasing caloric intake.

DNA. In control animals increased feeding resulted in an increase in total animal DNA and in liver, kidney, and heart DNA (table 3). Brain showed no increase in DNA. These increases are proportional to the increases in protein and weight. The weight/DNA and protein/DNA ratios remained constant. Hence cell number is increased while cell size remains normal.

In contrast, dwarf animals subjected to "overfeeding" showed no increase in total animal or individual organ DNA (table 3). Hence the weight/DNA and protein/DNA ratios increase. Cell number is unchanged —cell size is increased except in brain which is unaffected.

Longevity. The normal life span of pituitary dwarf mice in our laboratory is about 30 to 35 days. They gradually lose weight to about 5 g after weaning and die shortly thereafter. Dwarfs raised in a litter of 1 to 3 animals live to at least 65 days (animals were killed at that time) without any special care.[2] They also lose weight, but much more gradually, and at 65 days they still weigh more than normal dwarfs at their heaviest (about 7–8 g). Hence by

[2] All animals postweaning were raised with standard Rockland Rat and Mouse Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 2

Total organ protein content at weaning in normal and dwarf mice [1]

	8-10 animals/litter	1-3 animals/litter	8-10 animals/litter	1-3 animals/litter
	Normal control	"Overnourished" control	Normal dwarf	"Overnourished" dwarf
	mg	*mg*	*mg*	*mg*
Brain	70.0	69.4	49.0	47.0
Heart	13.0	17.0	4.0	10.0
Kidney	52.0	69.0	18.0	29.0
Liver	132.0	154.0	43.0	70.0
Whole animal	1200.0	1587.2	575.0	725.0

[1] Each value represents the average for 5 separate animals with a variation of less than 5% for any individual animal within a particular group. There is no overlap between groups except in the brain. All other organs show a statistically significant difference between each of the 4 groups.

TABLE 3

Total organ DNA content at weaning in normal and dwarf mice [1]

	8-10 animals/litter	1-3 animals/litter	8-10 animals/litter	1-3 animals/litter
	Normal control	"Overnourished" control	Normal dwarf	"Overnourished" dwarf
	mg	*mg*	*mg*	*mg*
Brain	0.77	0.80	0.50	0.50
Heart	0.14	0.17	0.07	0.07
Kidney	1.00	1.80	0.60	0.60
Liver	1.50	2.10	0.90	0.90
Whole animal	59.70	73.20	17.20	17.70

[1] Each value represents the average for 5 separate animals with a variation of less than 5% for any individual animal within a particular group. With the exception of brain there is no overlap of individual figures between normal control and "overnourished control" animals. All other differences are, therefore, statistically significant. However, there are no significant differences between the organs of normal dwarfs and "overnourished" dwarfs.

simply reducing nursing-group size, longevity of dwarf animals may be increased at least twofold. These animals never attain sexual maturity, although old enough, and are sterile.

DISCUSSION

The data in this study indicate that normal mice, like normal rats (7), respond to an increased dietary intake during the neonatal period by gaining weight faster. This gain is accompanied by an increased rate of cell division in all organs except brain.

In dwarf animals, however, the response to "overfeeding" is quite different. Although weight and total protein increase, DNA remains unchanged. These data may be interpreted to show an increase in individual cell size without increase in the rate of cell division or the ultimate number of cells.

Thus the premature cessation of cell division previously described in pituitary dwarf mice (3) is not affected by increasing the number of calories even though the animal gains considerable weight and deposits considerably more organ protein and adipose tissue. This lack of increase in cell number is unexpected because for at least the first six or seven days of life the pituitary dwarf animal grows normally. Either the increase in cell number in normal "overfed" animals begins after the first week or the pituitary dwarf is unable to respond in a normal manner to increased caloric intake even at a time when it is gaining weight at the same rate as its normal littermates. This abnormal response is characterized by increased deposition of adipose tissue and increased cell size within individual organs.

That weight and protein increased in dwarf animals while DNA content did not, suggests that pituitary hormone in vivo, along with its well-known effects on RNA and protein synthesis, may exert an independent role in the control of cell division.

These data do not answer the question of whether the cellular changes seen with both increased and decreased caloric intake are normally mediated through the anterior pituitary gland. They do, however, demonstrate an interrelation between anterior pituitary function, caloric intake, and cellular growth.

The increased longevity with increased calories is unexplained. This increased life span, however, should make it possible to use this species more efficiently as a model for studying long-term effects of congenital hypopituitarism.

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Antithyrotoxic Factor Content of Various Roughages and Cow Feces Based on Repression of Induced Liver Malic Enzyme in Thyrotoxic Rats [1,2]

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ABSTRACT The repression of liver malic enzyme induced by adding 0.025% iodinated casein (1% thyroxine (T_4) equivalent) to the basal diet was used to measure the antithyrotoxic factor (ATF) content of several roughages (flaked soybean hulls, alfalfa hay, corn silage and alfalfa-grass silages) and dried cow feces fed at the level of 15% in the diet of rats. Relative ATF units (100 units equivalent to the effect of adding 10% hemoglobin standard to the thyrotoxic diet) ranged from 77.6 to 116.3 in the roughages, soybean hulls being lowest and alfalfa-grass silage highest. The ATF content of the dried feces from cows fed alfalfa-grass silages was higher than that of the silages, ranging from 125.3 to 134.9 units.

The existence of an antithyrotoxic factor (ATF) in liver, yeast, soybean meal and other substances was first discovered by Ershoff in 1947 (1, 2). More recently Overby et al. (3) have conducted experiments on various sources of ATF. Our interest in the ATF content of roughages has been its possible relation to a decline in milk production frequently observed when abrupt changes in forage ration of lactating dairy cows were made (4) and to the increased thyroidal ^{131}I release rates in young cattle following similar changes in forage ration (5).

The original assays of ATF (1, 2) were based on the observations that substances such as liver, which have ATF activity, prevented the toxic effects of orally administered thyroxine measured by survival time and weight gain. Westerfeld and associates have developed assay procedures based on metabolic rate response (6, 7) and also on the response of rat liver α-glycerophosphate dehydrogenase. This study was based on the work of Tepperman et al. (8), who observed a seven-fold increase in rat liver malic enzyme resulting from feeding thyroxine. An assay procedure (9) was used in which the ATF content of different roughages and cow feces was determined by measuring the repression of liver malic enzyme induced by feeding thyroxine in the form of iodinated casein. Hemoglobin was used as the standard source of ATF based on the work of Richert et al. (6) who showed that both hemoglobin and liver were rich sources of ATF.

EXPERIMENTAL PROCEDURES

The basal group diet used was similar to the diet described by Westerfeld et al. (10) [4] except that a prepared vitamin diet fortification mixture was used. Thyrotoxicity was induced in the other groups of rats by feeding a thyrotoxic diet made by adding 0.025% iodinated casein (1% thyroxine (T_4) equivalent) to the basal diet. In the thyrotoxic diets used to determine the standard response curve, from 1 to 10% hemoglobin was substituted for equivalent amounts of casein so that a constant level of 30% protein was maintained. The roughages and cow feces to be tested for ATF activity were dried at 100° and incorporated at the 15% level in the experimental diets. They were substituted in the thyrotoxic diet for either casein,

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[1] From a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, The Ohio State University, Columbus, 1964.

[2] Approved for publication as Journal Article no. 76-67 by the Associate Director, Ohio Agricultural Research and Development Center, Wooster.

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[4] The basal diet consisted of the following: (in %) casein, 30; cottonseed oil, 10; salt mixture (no. 40, Steenbock and Nelson, J. Biol. Chem., 56: 355, 1923), 4; sucrose, 56; choline chloride, 0.1; and inositol, 0.05. To this the following vitamins were added (in g/45.5 kg) in the form of a vitamin diet fortification mixture in dextrose (obtained from Nutritional Biochemicals Corporation, Cleveland): α-tocopherol, 5; ascorbic acid, 45; choline chloride, 75; menadione, 2.25; vitamin A conc (crystalline vitamin A acetate, 200,000 U/g), 4.5; vitamin D conc (crystalline calciferol, D_2, 400,000 U/g), 5.0; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in mg/45.5 kg of diet) biotin, 20; folic acid, 90; and vitamin B_{12}, 4.54. Additional inositol and vitamin B_{12} were added to the vitamin mixture to provide the amounts indicated, equivalent to the amounts used by Westerfeld (10).

sucrose, or cottonseed oil, depending on their proximate composition so that all diets were similar with respect to the major nutrients.

Male weanling rats (Sprague-Dawley strain), weighing between 50 and 100 g, were fed the various rations for 11 days. They were killed on day 12 and their livers removed for malic enzyme assay using the method of Ochoa (11) as modified by Somasundaram et al. (9). The protein content of the liver extracts was measured by the method of Gornal et al. (12). Units of malic enzyme activity per milligram of protein in the liver extracts were calculated by the method of Ochoa (11). In the standard curve, zero to 100 ATF units was arbitrarily set to cover the range from zero to 10% hemoglobin added to the thyrotoxic diet. The hemoglobin dose response curve was linear in this range and was expressed by the following regression equation: ATF units = 152.93 − (3.14 × units of malic enzyme/mg of protein in liver extracts). A similar dose response curve (not used) was also obtained when liver residue was used as the ATF standards. Three rats were used to determine each percentage point in the hemoglobin ATF standard curve,

The remaining rats were divided into experimental groups of 10 each and fed the different diets in weighed amounts. Malic enzyme analyses were carried out the same day the rats were killed. An approximately sixfold increase in liver malic enzyme was measured between the 10 rats fed the basal diet and the 10 negative control rats fed the thyrotoxic diet containing no hemoglobin.

The different roughages and cow feces fed are indicated in table 1. Alfalfa-grass silage I had previously been shown to increase ^{131}I release rate when it was fed to heifers after abruptly changing from a ration of corn silage. Similarly, alfalfa-grass silage II was used because depressed dry feed intake of cows had resulted when their ration was abruptly changed from corn silage to legume-grass silage II. A cow being fed alfalfa-grass silage was fed 2 g of inorganic iodine (KI)/day for 5 days and a control cow was fed the same silage without KI. Their feces were collected after 3 days and oven-dried at 100°. The fecal collection from the control cow was designated as feces I and that from the KI-fed cow as feces II. Feces III was obtained from another cow fed alfalfa-grass silage II and no KI.

RESULTS AND DISCUSSION

Among the roughages tested (table 1), flaked soybean hulls were lowest in ATF activity and the alfalfa-grass silages highest. Of particular interest was the high ATF activity of the feces compared with that of the feeds tested. In this test 15% feces in the diet contained sufficient ATF activity to reduce the malic enzyme in the liver extracts of the thyrotoxic rats approximately to the basal level (7.32 ± 0.75 units). Baker et al. (13) have attributed the ATF activity of hemoglobin to its ability to bind thyroxine in the intestinal tract,

TABLE 1

Relative antithyrotoxic factor (ATF) content of different roughages and cow feces

Group no.[1]	Test material, 15% in ration	Avg units liver malic enzyme/mg protein	Avg relative ATF units [2]
1	Flaked soybean hulls	23.97 ± 2.71 [3]	77.6
2	Alfalfa hay	18.98 ± 2.00	93.3
3	Corn silage	16.86 ± 1.61	100.0
4	Alfalfa-grass silage I	14.13 ± 1.15	108.6
5	Alfalfa-grass silage II	11.67 ± 1.45	116.3
6	Cow feces I, no KI fed	5.74 ± 0.51	134.9
7	Cow feces II, KI fed	7.99 ± 0.85	127.9
8	Cow feces III, no KI fed	8.81 ± 0.63	125.3

[1] Ten rats/group.
[2] Calculated from the following linear regression equation: ATF units = 152.93 − (3.14 × units of malic enzyme per mg of protein in liver extracts).
[3] SE.

resulting in its excretion in the feces. At least a part of this effect was due to the iron porphyrin moiety of hemoglobin.

However, substances such as diacylated chitin, not closely related chemically to hemoglobin on the basis of solubility and stability, also were found to have ATF activity (13). Thus, it will require additional work to determine whether the ATF activity of cow feces is due to the development of some substances having ATF activity as the feed passes through the digestive tract or to concentration of the observed ATF activity in the feeds fed the cows.

It was thought that perhaps KI added to the ration of cows might result in the gastrointestinal synthesis of thyroactive substance(s). If this occurred it would appear in the feces, which when fed to thyrotoxic rats would alter the malic enzyme response to iodinated casein. However, in this experiment the addition of 2 g of KI/day to the diet of one cow fed alfalfa-grass silage did not markedly change the malic enzyme response or the ATF activity of the feces (feces II, table 1) compared with the control cow.

The relatively high ATF activity observed in these roughages would presumably minimize any effect of small amounts of thyroactive materials that might be ingested or synthesized in the digestive system as well as reduce the enterohepatic recycling of T_4 as shown by Ruegamer et al. (14, 15). Ruegamer and Wallace (14) also found that rats fed antithyrotoxic substances had more active thyroid glands, based on increased ^{131}I uptake and histological examination but the metabolic rates were the same as the controls. This indicated that the thyroid had to work harder in the presence of ATF in the diet to compensate for the greater fecal thyroxine excretion.

It is postulated that this effect of ATF on thyroid gland activity might become a limiting factor on milk production following a sudden change to a ration high in ATF content, until the thyroid has had time to adjust to the increased demand for T_4.

Pertinent to the problem of thyroidal effects of roughages is the observation of McLaren et al. (16) who found that 20% dried grass silage incorporated into the diets of rats increased their basal metabolic rates (BMR). These silages were found to be more active in increasing BMR after extraction with petroleum ether than with absolute alcohol. All the thyroid-stimulating activity of the silages could be removed by extraction with hot 80% ethanol. Hay made from grasses of similar botanical composition had no effect on BMR when fed at the level of 20% of the diet. These results suggest that perhaps both a thyroid-stimulating substance and an antithyroid substance may have developed in the silages since extraction by petroleum ether and absolute alcohol enhanced the thyroid-stimulating effect. The possible relation of ATF to these observations is of interest as a subject for future research.

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Interrelationship of Dietary Glycine, Methionine and Vitamin E in the Rat

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ABSTRACT The ability of methionine and vitamin E, singly or in combination, to counteract the effects of an excessive intake of glycine was investigated. Weanling male rats were fed a 10 or 20% isolated soybean (soy) protein or an 11% lactalbumin diet for 4 weeks. Excess glycine (5% of diet) caused a 20% growth depression with lactalbumin and a 38% depression with soy only in the absence of dietary vitamin E. DL-Methionine or vitamin E prevented liver necrosis as determined histologically. The rats fed the vitamin E-free diet showed accentuation in tooth discoloration when glycine was added to the diet. Growth of rats fed a 10% soy diet with 0.9% methionine was significantly reduced when glycine was added to this diet. No difference in growth was observed with glycine in the presence of 1.8% methionine. Glycine partially alleviated the severe toxic effect of 3.6% methionine. Significant differences in liver and kidney weights were noted. Factors found to influence the response of the rat to excess glycine, in addition to vitamin E and methionine, were the protein level and source. A definite relationship between glycine and the level of dietary methionine is indicated.

Adverse effects of dietary excesses of individual amino acids, including methionine, have been reviewed by Harper (1). Sauberlich (2) has studied the toxicity of 19 amino acids, including glycine, when fed to weanling rats as 5% of a low protein diet; each of the amino acids studied except alanine produced some degree of growth depression which could be partially or completely prevented by supplementing the diet with protein. Harper et al. (3) demonstrated that a large excess of an essential amino acid fed to weanling rats given a low protein diet depressed growth. When the diet was supplemented with protein or the limiting amino acid(s), the growth depression was alleviated.

Several investigators have shown that glycine (4–8) or arginine (6, 8) can alleviate the toxic (growth-depressing) effect of methionine; however, only limited study has been made of the ability of methionine to protect against excess glycine (4). A relationship between glycine and vitamin E has been suggested (4, 9). Our studies were designed to obtain additional information on the ability of methionine and vitamin E, singly or in combination, to counteract the effects of an excessive intake of glycine by rats fed different protein diets.

MATERIALS AND METHODS

Weanling male rats (Holtzman strain), 40 to 50 g, were housed in individual screen-bottom cages in a room maintained at 22 ±1°. Food and water were offered ad libitum. Food intakes and body weights were recorded weekly. The rats were fed a purified protein diet with or without added vitamin E, DL-methionine and glycine for 4 weeks. As sources of protein, the diets contained 10% or 20% of isolated soybean (soy) protein [1] or 11% of lactalbumin.[2] The crude protein content (Kjeldahl N × 6.25) of the 10 and 20% soy diets and the lactalbumin diet were 9.2, 18.4 and 9.2%, respectively. Glycine was used at a 5% level since, in a preliminary experiment, higher levels (6, 8 and 10%) failed to produce significantly greater effects with either protein. Additions to the diets were made at the expense of sucrose. All diets had the following percentage composition: stripped lard,[3] 10; mineral mix (10), 4; vitamin mix (without vitamin A and vitamin E) (11), 4. Vitamin A in sucrose was added to supply 200 IU/100 g of diet. Vitamin E, when a variable, was administered

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[1] C-1 Assay Protein, Skidmore Enterprises, Cincinnati.

[2] The DL-methionine, glycine and lactalbumin were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Distillation Products Industries, Rochester, New York.

TABLE 1

Effect of glycine, methionine and vitamin E on rats fed a 10% soy protein diet for 4 weeks [1]

Group no.	Dietary supplement [2]		Mortality [3]	Avg tooth color index [4]	Avg body wt gain	Food efficiency	Liver wt	Kidney wt	GOT [4]
	Vitamin E	Glycine, 5%							
					g/day	*g gain/g food*	*% body wt*	*% body wt*	*units/ml*
					No methionine added				
1	−	−	3/15	3.5 ± 0.3 [5]	0.8 ± 0.1 [6]	0.13 ± 0.01 [6]	5.40 ± 0.16 [6]	1.12 ± 0.04 [6]	—
2	+	−	0/19	3.9 ± 0.1	0.8 ± 0.1	0.11 ± 0.01	4.70 ± 0.13	1.03 ± 0.03	90
3	−	+	3/15	2.1 ± 0.5	0.5 ± 0.1	0.09 ± 0.01	5.14 ± 0.23	1.33 ± 0.06	—
4	+	+	0/20	3.8 ± 0.1	0.9 ± 0.1	0.14 ± 0.01	4.44 ± 0.07	1.17 ± 0.03	70
					0.3% methionine added				
5	−	−	0/9	3.8 ± 0.1	1.5 ± 0.1	0.21 ± 0.01	4.89 ± 0.08	0.81 ± 0.02	—
6	+	−	0/13	3.8 ± 0.1	1.8 ± 0.1	0.23 ± 0.01	5.14 ± 0.08	0.83 ± 0.02	81
7	−	+	0/10	3.8 ± 0.1	1.5 ± 0.1	0.23 ± 0.01	4.47 ± 0.12	1.02 ± 0.02	—
8	+	+	0/15	3.8 ± 0.1	1.5 ± 0.1	0.22 ± 0.01	4.59 ± 0.06	0.98 ± 0.02	56
					0.9 methionine added				
9	−	−	0/10	3.4 ± 0.2	1.9 ± 0.1	0.23 ± 0.01	5.20 ± 0.11	0.87 ± 0.03	—
10	+	−	0/15	4.0 ± 0.1	1.9 ± 0.1	0.23 ± 0.01	5.16 ± 0.10	0.93 ± 0.02	88
11	−	+	0/10	3.7 ± 0.1	1.3 ± 0.1	0.21 ± 0.01	4.29 ± 0.13	1.07 ± 0.02	—
12	+	+	0/14	3.6 ± 0.1	1.4 ± 0.1	0.20 ± 0.01	4.70 ± 0.12	1.06 ± 0.02	68
					1.8% methionine added				
13	+	−	0/5	—	1.2 ± 0.1	0.18 ± 0.01	5.27 ± 0.16	1.18 ± 0.03	210
14	+	+	0/5	—	1.2 ± 0.1	0.19 ± 0.01	5.17 ± 0.17	1.21 ± 0.04	172
					3.6% methionine added				
15	+	−	0/4	—	−0.2 ± 0.1	−0.04 ± 0.01	4.46 ± 0.12	1.60 ± 0.07	>216
16	+	+	0/5	—	0.6 ± 0.1	0.13 ± 0.02	4.46 ± 0.20	1.42 ± 0.02	144

[1] Diet was equivalent to 9.2% of protein by analysis (Kjeldahl, N × 6.25).
[2] Vitamin E, when a supplement, administered as *dl*-α-tocopheryl acetate to supply 3 mg vitamin E twice weekly.
[3] Number of deaths/number of rats initially started.
[4] Average glutamic-oxaloacetic transaminase values for pooled plasma for 5 rats/group.
[5] Average ± SE of mean.
[6] Average ± SE of mean; values reported to the nearest tenth.

orally in the form of *dl*-α-tocopheryl acetate in corn oil to supply 3 mg vitamin E twice weekly. In the other experiments, *dl*-α-tocopheryl acetate (0.02%) mixed in sucrose was supplied in the diet.

Tooth pigmentation was rated at the end of the 4-week experimental period (12). Rats were then killed and blood samples were obtained as previously described (13). Hemoglobin was determined by the cyanmethemoglobin method (14) on pooled samples of fresh whole blood from each group. Plasma was obtained by centrifugation at 5°. Glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase were determined on pooled samples of fresh plasma by the colorimetric Sigma-Frankel procedure.[4] The liver, kidneys, testes and spleen of each rat were weighed. Livers were examined macroscopically for massive necrosis and then fixed in 10% buffered formalin for histological examination. Sections were stained with hematoxylin and eosin. The methionine, a synthetic product, was analyzed for selenium colorimetrically (15). Data were analyzed statistically by Student's *t* test (16).

RESULTS AND DISCUSSION

Effect of vitamin E. Table 1 shows that at 4 weeks, rats fed the 10% soy protein diet without vitamin E (E-free group), with or without 5% of glycine (groups 1 and 3), lost some of the normal pigmentation of the upper maxillary incisors and exhibited increased mortality, indicating vitamin E deficiency. Average tooth color index of the vitamin E-free group receiving glycine was 2.1 on an arbitrary scale ranging from zero for completely depigmented to 4 for no tooth decoloration. The index of all the other groups was 3.4 or higher. Glycine thus accentuated the dental depigmentation in vitamin E deficiency. Histological examination confirmed the necrosis seen macroscopically in the livers of rats in the vitamin E-free (fig. 1A) or vitamin E-free plus glycine groups (same architecture as in fig. 1A). The livers of rats receiving supplemental vitamin E had a normal architecture (fig. 1B).

Data on body weight gain, food efficiency and organ weights are shown in table 1. The addition of 5% of glycine to the unsupplemented vitamin E-free diet (group 3 vs. 1) reduced weight gain and food efficiency. Weight gain and food efficiency were not increased by the addition of vitamin E alone (group 2 vs. 1), but were significantly improved by vitamin E in combination with glycine ($P < 0.001$) as compared with the group receiving glycine as the only supplement (group 4 vs. 3). Vitamin E did not affect growth when methionine was added to the diet at the 0.3 and 0.9% levels, either with or without added glycine (groups 6, 8, 10, 12 vs. 5, 7, 9, 11, respectively).

Effect of methionine supplementation and protein level. Methionine at the 0.3 and 0.9% levels significantly improved growth and food efficiency ($P < 0.001$) of the vitamin E-free group, with or without added glycine (table 1, groups 5 and 9 vs. 1; 7 and 11 vs. 3). Growth and food efficiency of the rats fed 1.8% of methionine without added glycine were significantly lower ($P < 0.01$) than the responses of rats fed 0.9% (group 13 vs. 10). Methionine at the 3.6% level (group 15 vs. 2) severely depressed growth ($P < 0.001$). Glycine did not reduce growth of the group fed 1.8% of methionine (group 14 vs. 13) and markedly improved ($P < 0.001$) growth of the group fed 3.6% of methionine (group 16 vs. 15). However, the toxic effect of the excess methionine was only partly alleviated.

When 0.9% of methionine was added to a 10% soy protein diet, glycine depressed growth of rats (group 12 vs. 10). Glycine did not affect growth of rats fed 1.8% of methionine (group 14 vs. 13), indicating that the response to excess glycine is partially related to the level of dietary methionine. No liver necrosis was observed in any rats receiving supplemental methionine with or without glycine (fig. 1C). A low level of selenium has been shown to prevent necrosis (17); however, analysis of the methionine used in this study showed no detectable selenium.

Organ weights, as percentage of body weight, were not changed by the addition of excess glycine to the unsupplemented soy diet (table 1, group 3 vs. 1). However,

[4] Sigma Chemical Company, St. Louis, Tech. Bull. no. 515, September 1964.

Figure 1A

Fig. 1 Histology of livers of rats fed (*A*) unsupplemented 10% soy protein diet. Areas of coagulative necrosis infiltrated by polymorph leukocytes can be seen close to the central vein. There is a slight extravasation of red blood cells; (*B*) soy protein diet supplemented with *dl*-α-tocopheryl acetate (3 mg in corn oil twice weekly). Liver has a normal architecture with intact hepatic cords radiating from the central vein. Hepatic cells show a very slight vacuolation; (*C*) soy protein diet supplemented only with DL-methionine (0.3%). The architecture is preserved. Note the radiating hepatic cords and portal triads from the central vein. There is a slight-to-moderate vacuolation of the parenchymal cells (H & E. × 136).

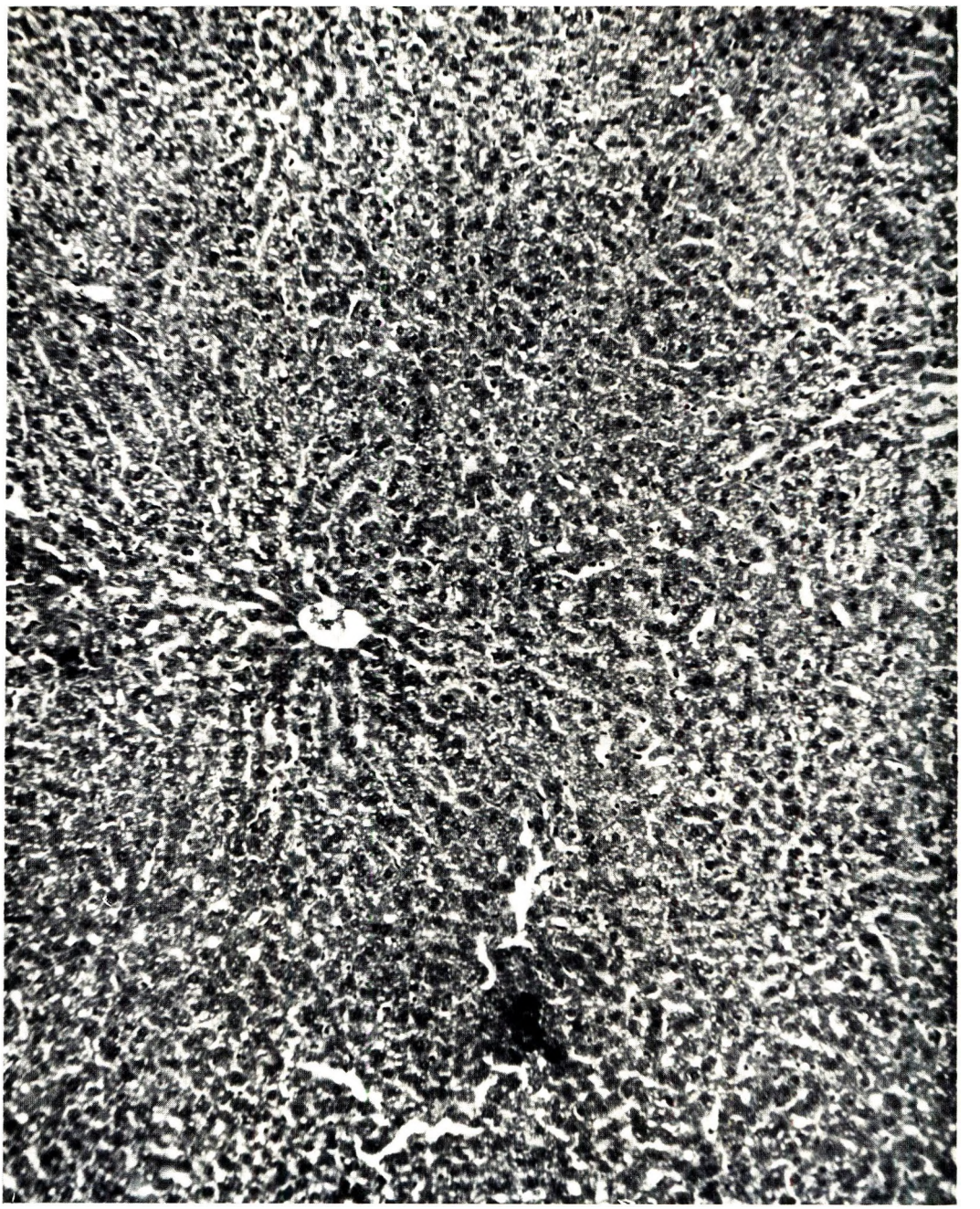

Figure 1B

the addition of glycine to the 0.3 and 0.9% of methionine-supplemented vitamin E-free diets (groups 7 vs. 5, 11 vs. 9) reduced liver size ($P < 0.01$) but increased kidney size ($P < 0.01$). Group 15, the rats receiving the highest level of methionine (3.6%), had the largest kidneys ($P < 0.001$) of all animals. The addition of glycine (group

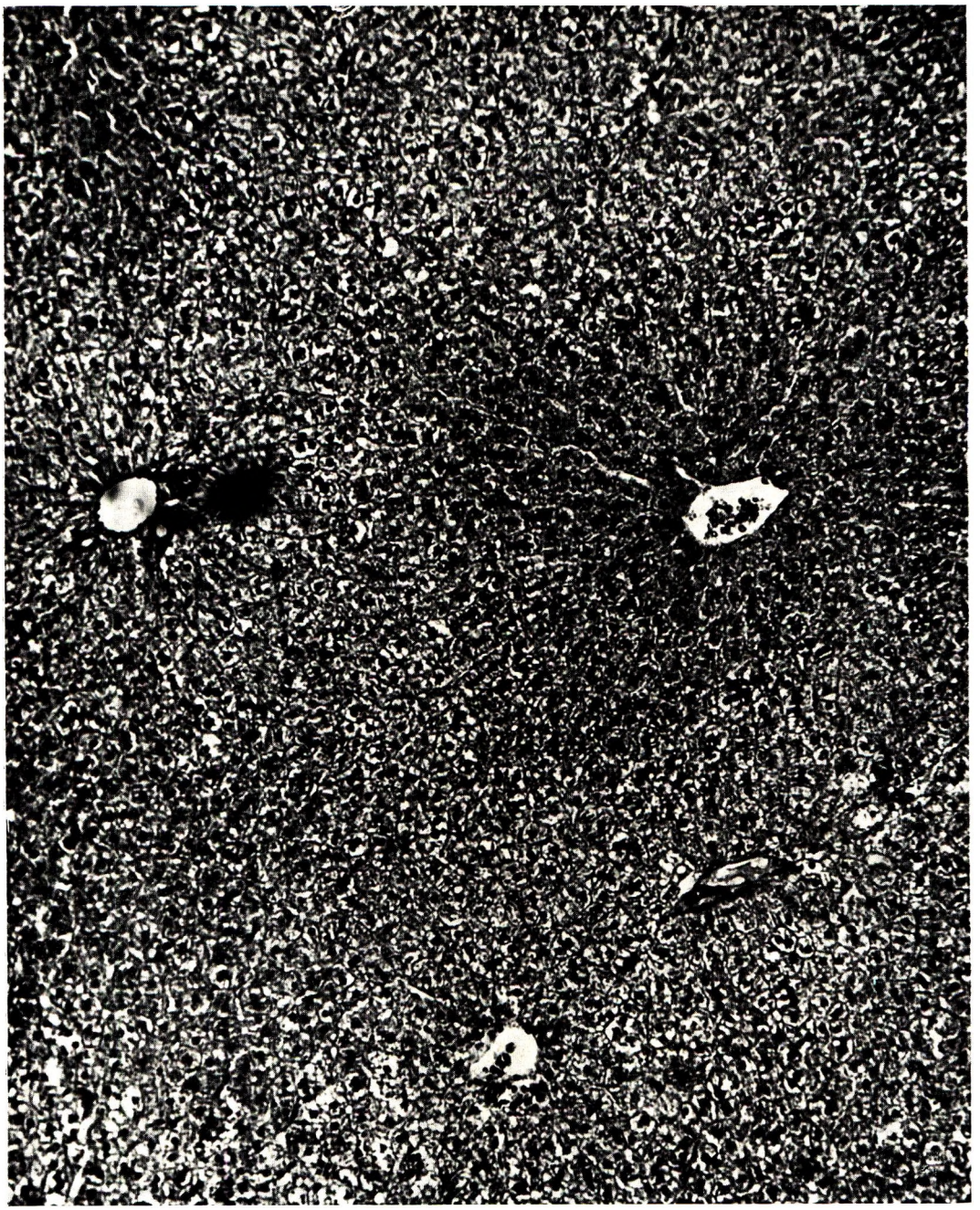

Figure 1C

16) significantly reduced kidney size ($P < 0.01$). Other workers (6) have also observed that glycine counteracts kidney hypertrophy caused by excess methionine. Spleen weights were significantly decreased when vitamin E was added to the diet without glycine (0.30 ± 0.02 to 0.25 ± 0.01, $P < 0.05$) or with glycine (0.36

TABLE 2

Effects of glycine and DL-*methionine on the growth and food efficiency of weanling rats fed a 20% soy protein diet for 4 weeks*

Diet supplements [1]		Avg body wt gain	Food efficiency
Glycine, 5%	Methionine		
	%	*g/day*	*g gain/g food*
−	—	3.9 ± 0.3 [2]	0.32 ± 0.01
+	—	3.2 ± 0.3	0.31 ± 0.01
−	0.3	5.7 ± 0.3	0.44 ± 0.02
+	0.3	4.7 ± 0.2	0.40 ± 0.01
−	0.9	5.5 ± 0.2	0.45 ± 0.01
+	0.9	4.5 ± 0.3	0.41 ± 0.01
−	1.8	4.0 ± 0.2	0.40 ± 0.01
+	1.8	4.2 ± 0.2	0.40 ± 0.01
−	3.6	1.0 ± 0.1	0.20 ± 0.02
+	3.6	2.9 ± 0.1	0.35 ± 0.01

[1] All diets contained vitamin E (0.02% of *dl*-α-tocopheryl acetate).
[2] Average ± SE of mean for 5 rats/group.

± 0.02 to 0.23 ± 0.01, $P < 0.001$). No differences in testes weights were observed.

Activities of glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase were increased in the livers of rats fed excess methionine (7). Table 1 shows that the glutamic-oxaloacetic transaminase activity in the plasma of rats fed glycine was consistently lower than in controls. Our results for the effect of glycine and excess methionine on glutamic-oxaloacetic transaminase values are in agreement with the work of Klain et al. (7). These workers also observed increased glutamic-oxaloacetic transaminase in the livers of rats fed a high level (4%) of methionine, and reduced glutamic-oxaloacetic transaminase activity with 4% of glycine. The glutamic-pyruvate transaminase activity or hemoglobin levels of rats fed the various diets were not consistently different from controls.

Effect of source and level of protein. To determine the influence of the level of dietary protein on the previously observed effects of methionine and glycine, soy protein was doubled. Table 2 shows the growth and food efficiency data for rats fed varying levels of methionine with a 20% soy protein diet for 4 weeks. The growth reduction with glycine was more pronounced at the 20% level than at the 10% level of protein, especially with 0.3% added methionine. This effect of protein level was unexpected since Sauberlich (2) found that adverse effects from large additions of some amino acids were less severe when the dietary protein was increased. With 20% of soy, glycine significantly reduced the rate of gain ($P < 0.01$) and lowered the food efficiency at the 0.3% and 0.9% levels of methionine supplementation but improved growth and food efficiency with 3.6% of methionine ($P < 0.001$). Methionine (0.3% or 0.9%) significantly improved growth ($P < 0.01$) over the unsupplemented basal diet; but 3.6% methionine severely depressed growth ($P < 0.001$).

Figure 2 shows that lactalbumin, in general, gave a dose response curve similar to soy when glycine was added to diets supplemented with graded levels of methionine (0.15, 0.3, 0.9, 1.8 and 3.6%). An important difference is that at the 0.3% level of supplemental methionine, glycine caused the greatest reduction in growth with lactalbumin or 20% of soy, but with 10% of soy the greatest reduction occurred at the 0.9% level of methionine. The lactalbumin and soy basal diets supplied 0.32 and 0.38% (calculated) of glycine, respectively, and 0.61 and 0.15% (calculated) of sulfur amino acids (methionine and cystine), respectively. If the total percentage of dietary sulfur amino acids were plotted versus average gain, the maximum point of depression due to glycine with both proteins would be at approximately 1% total sulfur amino acids. The observation that excess glycine causes a greater growth depression in weanling rats fed

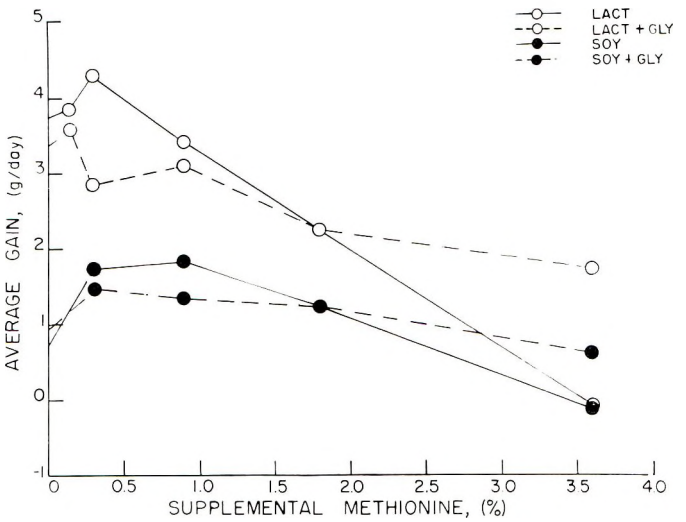

Fig. 2 The average 4-week body weight gain of weanling rats fed purified lactalbumin (lact) or soy protein diets supplemented with DL-methionine with or without 5% of glycine. The diets supplied 9.2% of protein (N × 6.25) and contained 0.02% of vitamin E (*dl*-α-tocopheryl acetate). Each point represents the mean value for at least 7 rats.

lactalbumin than with soy protein agrees with the findings of Sauberlich (2).

Our results indicate an interaction between glycine and methionine; however, the mechanism involved is yet to be determined. Harper (1) has recently reviewed specific interactions between pairs or small groups of other amino acids.

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Requirements of the Female Rat for Linoleic and Linolenic Acids[1]

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ABSTRACT To determine the requirement of the female rat for essential fatty acids, weanlings were fed a fat-free diet supplemented with highly purified ethyl linoleate or ethyl linolenate at several levels. Weight gain, food efficiency, dermal symptoms of deficiency, and fatty acid composition of liver, heart, erythrocyte, and plasma lipids were determined and comparisons made with male rats from earlier experiments. Minimum linoleate requirement of the female rat was estimated as 0.5% of calories, of male rats, as 1.3%. The requirement for linolenate was estimated to be 0.5% of calories for both males and females. The degree of unsaturation of tissues was found to be different for the 2 sexes. Female tissues had lower proportions of total saturated and monoenoic fatty acids and higher proportions of the more unsaturated, long-chain metabolites of oleic, linoleic and linolenic acids. Calculations of the number of double bonds per fatty acid showed that the fatty acids of female tissues contained 1.3 to 1.6 times more double bonds than those of male rats.

Burr and Burr (1), in their earliest description of fat deficiency syndromes in the rat, observed a sex difference in growth which suggested that the female was less disadvantaged than the male when fat was excluded from the diet. Later workers, all of whom used growth as their principal criterion of adequacy, estimated the essential fatty acid (EFA) requirement per day for the female rat to be 20 mg by Martin (2) and by MacKenzie et al. (3), 40 mg by Burr et al. (4), 42 mg by Hume et al. (5, 6), and 100 mg by Turpeinen (7). More recently, Deuel and his group (8), also using growth as a criterion, estimated the EFA requirement to exceed 200 mg per day for male rats and to be 10 to 20 mg for females. Greenberg et al. (9) increased the latter estimate to 50 mg and also observed that when dietary linoleate was increased to 100 mg per day, growth in the female was depressed.

Studies in this laboratory have established the linoleate requirement of the young male rat to be approximately 1% of total caloric intake (10) or approximately 40 mg per day. Among the biochemical responses, the principal parameter of adequacy was the triene-to-tetraene ratio measured from the fatty acid composition of the tissues and gain in weight was a supporting criterion of adequacy.

The purpose of the present experiment was to determine the EFA requirement of the young *female* rat using biochemical parameters and to compare requirements and fatty acid metabolism of the 2 sexes when either linoleate or linolenate was the sole fatty acid supplement to a fat-free basic diet. Previous experiments in this laboratory with male rats (11–13) were duplicated as closely as possible.

EXPERIMENTAL

One hundred and twenty-three, 21-day-old female rats of the Sprague-Dawley strain were fed a basic fat-free ration[3] until they were 30 days of age. They were then distributed at random into 20 groups. One group of 9 control animals was fed the basic fat-free ration without supplements. Ten groups of 6 animals were fed daily supplements of highly purified ethyl

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[3] The composition of the semipurified fat-free diet was as follows: (in percent) vitamin-free casein, 18; sucrose, 74; Wesson salt mixture, 4; cellulose (Alphacel), 4; and vitamin mixture. The vitamin mixture contained: (in mg/kg of diet) thiamine·HCl, 30; riboflavin, 30; pyridoxine·HCl, 8; DL-calcium pantothenate, 100; niacin, 100; *i*-inositol (meso), 220; *p*-aminobenzoic acid, 75; folic acid, 1; biotin, 0.20; vitamin B_{12}, 0.05; vitamin A acetate, 4; vitamin D_2, 4; vitamin K_3, 2; *dl*-α-tocopherol, 280; and choline chloride, 1000. Salt mixture: Wesson, L. G., Science, 75: 339, 1932. One kilogram of the diet was mixed with 100 ml of a solution of the vitamin A acetate and α-tocopherol in ether and the ether was evaporated. All components of the basic diet except sucrose were purchased from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Mean weight gain, food efficiency, and dermal symptoms of deficiency of female rats fed fat-free diet supplemented with ethyl linoleate or linolenate

Diet supplement	No. of animals	Mean wt gained	Food efficiency	Dermal score [1]
% of calories		*g*	*g gain/100 g food*	
Linoleate				
None	9	114 ± 18 [2]	10.7	4.2 ± 0.4 [2]
0.01	6	125 ± 11	11.7	3.5 ± 0.7
0.02	6	118 ± 27	10.9	3.7 ± 0.8
0.05	6	126 ± 16	11.8	4.0 ± 0.5
0.11	6	125 ± 18	11.7	2.8 ± 1.3
0.22	6	136 ± 24	12.0	2.2 ± 0.7
0.34	6	132 ± 20	11.4	1.4 ± 0.6
0.68	6	134 ± 14	12.8	1.0 ± 0.4
1.18	6	147 ± 16	14.3	1.2 ± 0.5
1.95	6	143 ± 22	13.1	1.6 ± 0.6
3.85	6	135 ± 19	13.0	3.2 ± 0.8
Linolenate				
0.01	6	126 ± 12	11.2	4.0 ± 0.6
0.02	5	137 ± 9	12.5	4.3 ± 0.3
0.05	6	134 ± 24	11.8	3.5 ± 0.8
0.11	6	124 ± 8	11.6	3.6 ± 0.9
0.22	6	125 ± 20	11.5	4.5 ± 0.5
0.54	6	133 ± 18	12.4	4.2 ± 0.8
1.00	6	134 ± 22	12.8	2.9 ± 0.7
1.98	5	134 ± 20	13.3	3.5 ± 0.6
3.91	6	140 ± 18	13.3	4.2 ± 0.8

[1] For explanation of scoring system, see (14).
[2] SD.

linoleate, 18:2ω6,[4] in amounts varying from 0.1% to 3.9% of total calories. Nine groups of 6 rats were fed daily supplements of highly purified ethyl linoleate, 18:3ω3,[5] in amounts varying from 0.01% to 3.9% of total calories, as listed in table 1. Fatty acid supplements were fed orally by microsyringe, each day, for 100 days. The amount fed was calculated as percentage of calories of basic diet consumed by each animal on the previous day. Weights were recorded weekly.

Purity of 18:2ω6, as checked by gas-liquid chromatography (GLC), was greater than 99.9% and of 18:3, 99.7%. Infrared spectroscopy showed that the 18:2ω6 contained 1.1% of the *trans* isomer and linolenate, 25%, using methyl elaidate as the standard.

After 100 days of supplementation, the dermal symptoms of EFA deficiency of each animal were recorded, using a system previously described (14). The animals were anesthetized with ether and killed by exsanguination. Blood plasma and erythrocytes were pooled for each group. The erythrocytes were washed twice with saline and stored in chloroform:methanol (2:1). Hearts and livers were blotted dry and stored in saline. All tissues were kept in a freezer at −20° until analysis was performed. The tissues were homogenized with 20 volumes of chloroform:methanol (2:1) and the lipids transesterified by refluxing with 30 volumes of a 5% solution of hydrogen chloride in methanol. All procedures were conducted under nitrogen.

The methyl esters were analyzed by GLC using a Research Specialties gas chromatograph with argon ionization detector. A 210-cm glass column of 5 mm i.d., packed with 20% ethylene-glycol-succinate polyester plus 2% phosphoric acid coated on Gaschrom P, 80 to 100 mesh, was used. The flow rate was 60 ml argon/minute at an inlet pressure of 700.3 g/cm^2 (10 psi). The inlet heater was kept at 250° and the detector cell at 235°. Temperature of the column was 195°. Two recorders, 1.0 mv and 10.0 mv full range, were connected in parallel which permitted measurement of

[4] Ethyl linoleate and ethyl linolenate were purchased from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minnesota, 55912.
[5] See footnote 4.

all tissue fatty acids from a single injection. Individual esters were identified using authentic standards and using a beef testis lipid extract for which all fatty acids had been characterized by ozonolysis and reduction (15). Quantification was by triangulation, and fatty acids data are reported as area percent.

RESULTS AND DISCUSSION

Weight gain, food efficiency and dermal symptoms of fat deficiency. The effects of graded levels of dietary ethyl linoleate on mean weight gain, food efficiency and dermal symptoms deficiency of female rats are presented in table 1. Females fed increasing amounts of linoleate gained weight progressively until a maximum was reached at approximately the same intake of linoleate (1.2% of calories) as observed (11) for males (1.3% of calories). Feeding more linoleate resulted in a lesser weight gain in both sexes.

Maximum food efficiency for females was 14.3 g/100 g food when 1.2% of calories of linoleate were fed. For males (11) a maximum of 26.8 g/100 g food was attained when the highest level (4.9% of calories) was fed, but the increase beyond the 1.3% level of calories of supplementation was minor.

Ethyl linolenate permitted weight gain in both sexes at about the same level as linoleate (table 1). Maximal weight gain occurred in males fed 1.4% of calories of linolenate, beyond which weight gain tended to decrease (11). Feeding females more than 0.5% of calories of linolenate resulted in only minor additional gain. In both sexes food efficiency tended to increase with increasing amounts of dietary linolenate, reaching a plateau at 3.6% of calories of 18:3ω3 for males and 2.0% of calories for females. All female rats fed linolenate and those fed less than 0.05% linoleate had mean dermal scores indicative of EFA deficiency. The lowest mean dermal score for females occurred when linoleate was fed at 0.7% of calories. Of the male rats (11), only those fed linoleate at the 0.05% level of calories or less and those fed a high level of linolenate (9.4% of calories) had scores indicative of EFA deficiency. Linolenate was more beneficial to the male than to the female for weight gain and maintenance of dermal integrity, but both sexes converted food to body tissue more efficiently when higher levels of linolenate were fed.

Fatty acid composition of tissues. Those fatty acids of liver lipids showing the greatest responses to diet were plotted as a function of dietary linoleate or linolenate (fig. 1). Curves representing previously published data for males (11) were drawn as broken lines to facilitate comparison. Analyses of fatty acid from heart, erythrocyte and plasma lipids were also made but are not tabulated because the effects noted in these tissues are qualitatively similar to those found in liver.

Deficient rats. The biochemical changes characteristic of EFA deficiency in males were observed in females as well. There were lower levels of saturated and of the ω6 fatty acids and higher levels of ω9 fatty acids and of 16:1ω7 than in supplemented animals. Tissue lipids of deficient male and female rats differed primarily in degree of unsaturation, those of females being more unsaturated. These data are presented in figure 1 as the points on the ordinate.

The concentrations of 20:4ω6 and 22:5ω6 were three and six times higher, respectively, in the females than in the males, whereas the level of 18:2ω6 was similar for both sexes. The concentration of 20:3ω9 was twice as high in liver lipids of deficient females as in males, but the latter had more 18:1ω9 than the females, suggesting that in the female chain elongation and desaturation of oleic acid are effected more efficiently than in the male. The concentrations of 22:6ω3 in liver and heart lipids were alike, 2% in female and 0.3% in male.

In the 3 tissues studied, liver, heart and erythrocytes, there were no sex differences in total ω9 content, but 20:3ω9 was consistently more abundant in female tissues and 18:1ω9 in male tissues. The livers of deficient females had approximately twice as much 18:0 and half as much 16:0 as in the males. Total saturated fatty acid content of male livers was considerably higher than that of females (38% vs.

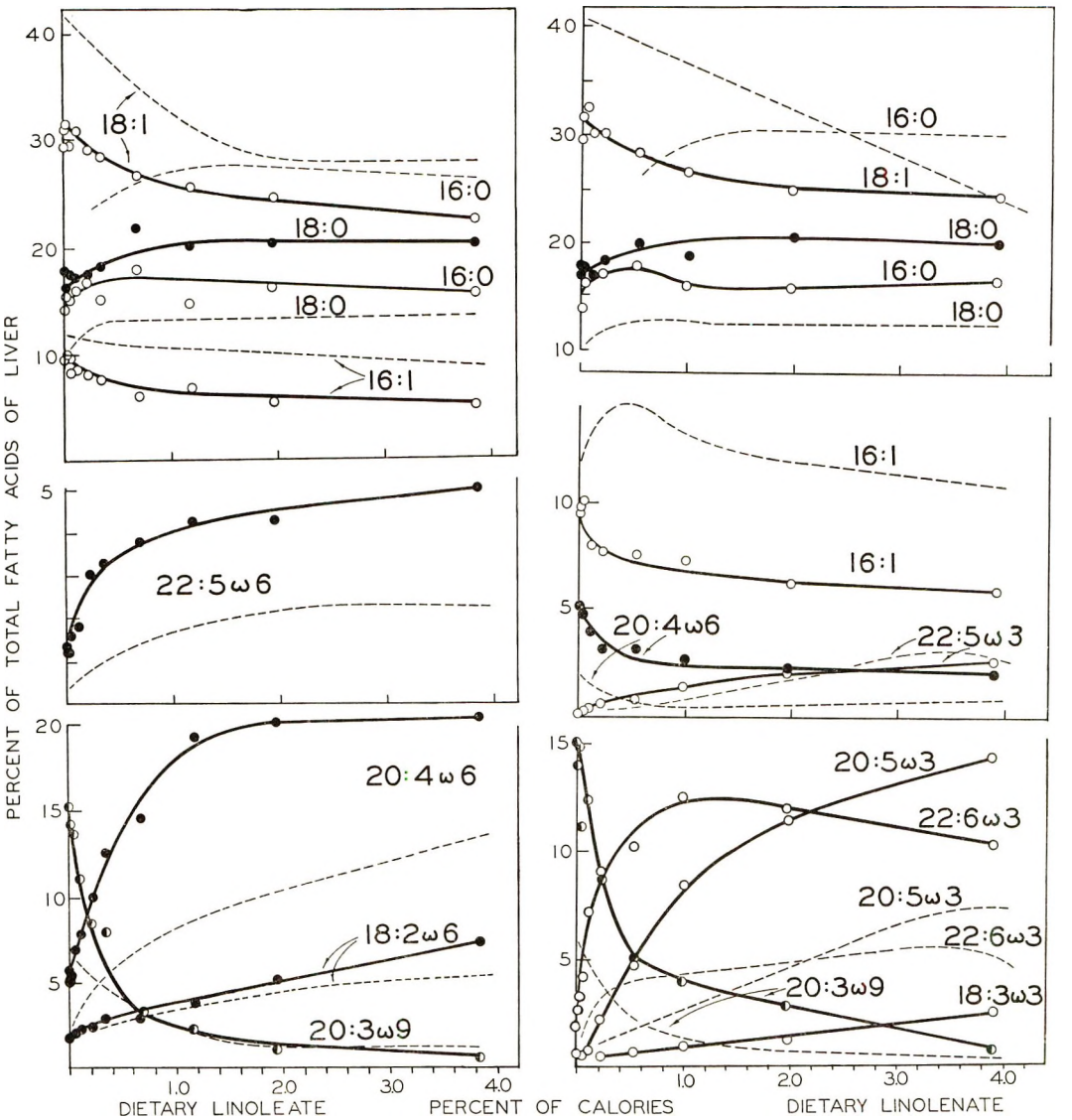

Fig. 1 Fatty acid composition of total liver lipids from rats fed different levels of ethyl linoleate or linolenate. Solid lines with points represent data from female rats. Dashed lines represent data from male rats (redrawn from Mohrhauer and Holman (11)).

33%). The sums of the 16:1ω7 and 18:1ω9 were also higher in males than in females (52% vs. 39%). In the deficient male, in which fewer long-chain highly unsaturated acids are synthesized than in the female, 16:0 is also apparently substituted for 18:0 as a partial alleviation of the need for acids of low melting point. The greater tendency for saturated and monounsaturated acids to accumulate in liver lipids in the male may be the primary cause of their higher requirement for dietary polyunsaturated acids (see later).

Linoleate-fed rats. When linoleate supplemented the basic fat-free diet, members of the linoleate family of acids showed the greatest increases in liver lipids, whereas the oleate family was decreased most sig-

nificantly. At the highest dietary levels of 18:2ω6 the concentration of all ω6 acids had not reached a plateau in liver lipids of female rats. In female heart lipids, however, 20:4ω6 reached a plateau at 1.2% of calories. In the erythrocytes, the level of 20:4ω6 decreased from 26% to 21% with dietary intakes higher than 0.7% of calories. In the plasma of female rats, the level of 20:4ω6 continued to increase sharply over the entire range of dietary 18:2ω6. At each level of 18:2ω6 fed, the concentration of 20:4ω6 and 22:5ω6 was considerably greater in all tissue lipids of females than of males. For example, the level of 20:4ω6 was 1.5 to 2 times higher in erythrocytes, 1.5 to 3 times higher in the liver, and 2 to 4 times higher in the heart than in corresponding male tissues.

When 18:2ω6 was fed there was a sharp and progressive decrease in the levels of 20:3ω9 and 18:1ω9 in the livers of both sexes. In all dietary groups male rats maintained considerably higher levels of 18:1ω9. On supplements of 0.7% of calories and less, levels of 20:3ω9 were higher in liver lipids of females, but on intakes of 1.2% of calories or higher they were lower than in livers of males. At low levels of dietary linoleate the 20:3ω9 decreased more abruptly in female liver, but 18:1ω9 decreased more sharply in male liver; 16:1ω7 was approximately 2% higher in male liver than in female liver at all dietary levels of 18:2ω6 and tended to decrease with increasing intake of linoleate in both sexes. These observations suggest that in the female rat, the chain is lengthened and fatty acids are dehydrogenated more readily than in the male.

Saturated fatty acids were affected much less than the ω6 and ω9 fatty acids by change in dietary 18:2ω6. In male livers the level of 18:0 increased over the whole range of dietary 18:2ω6, whereas in the female a plateau was reached at 1.2% of calories. In all dietary groups male livers contained more 14:0 and 16:0 than livers of females. In heart lipid the content of individual saturated fatty acids showed no consistent trends in either sex regardless of dietary supplement. For each level of dietary supplement, the content of 18:0 was higher and of 16:0 was lower in females than in males. However, the sum of saturated acids was always greater for the male. In the erythrocytes there were no consistent trends in levels of the saturated fatty acids with dietary 18:2ω6. These observations suggest that shorter-chain acids may substitute partially in the male for the polyunsaturated acids present more abundantly in the female.

Linoleate requirement. The ratios of 20:3ω9/20:4ω6, which have been used as a parameter for assessing the EFA requirement (10), were calculated for each tissue (table 2). A triene-to-tetraene ratio below 0.4 corresponds to normal EFA status in male rats. For female livers, the value of 0.4 was reached at a linoleate intake of 0.5% of calories, whereas for male livers

TABLE 2

Triene-to-tetraene ratios of tissues of rats fed ethyl linoleate

Linoleate		Tissues						
		Liver		Heart		Erythrocytes		Plasma
Male	Female	Male	Female	Male	Female	Male	Female	Female
% of calories								
None	none	3.65	2.67	5.24	2.00	2.28	1.30	3.69
0.01	0.01	3.30	2.36	3.21	1.67	1.99	1.19	2.96
0.02	0.02	3.63	2.82	3.54	1.96	1.78	1.20	2.45
0.05	0.05	2.98	1.99	2.69	1.35	1.64	0.88	3.03
0.10	0.11	1.93	1.40	1.73	0.88	1.03	0.63	1.67
0.18	0.22	1.62	0.84	1.44	0.43	0.76	0.36	0.52
0.32	0.34	1.18	0.63	1.39	0.27	0.59	0.25	0.47
0.61	0.68	0.63	0.22	0.18	0.14	0.25	0.09	0.18
1.26	1.18	0.40	0.11	0.18	0.04	0.12	0.04	0.07
1.79	1.95	0.16	0.05	0.06	0.03	0.05	0.02	0.03
4.87	3.85	0.01	0.02	0.04	0.02	0.03	0.01	0.01

it was reached at an intake of 1.3% of calories. The value of 0.4 for female hearts occurred at 0.2, for male hearts at 0.5; female erythrocytes at 0.2, for male erythrocytes at 0.5% of calories. For each tissue, the minimal requirement for linoleate of male rats was 2.5 times greater than that for females. For both sexes, the amount of dietary linoleate required to produce a normal triene-to-tetraene ratio in liver was 2.5 times greater than that for heart or erythrocytes. The minimal requirement of female rats, based on the triene-to-tetraene ratio of plasma was 0.5% of calories of linoleate. Thus, all these metabolic requirements are met by 1.25% of calories for males and 0.5% of calories for females.

Optimal range of intake of 18:2ω6. When the dose-response curves for all relevant acids of various tissues of female rats were examined, the optimal range of dietary 18:2ω6 was found to be rather narrow. Supplements greater than 0.68% of calories produced an increase in dermal scores (table 1) and a decrease in level of 20:4ω6 in the erythrocytes. Supplements greater than 1.2% of calories brought about a decrease in weight gain and food efficiency (table 1) despite the more than adequate amount of tocopherol in the diet. At this level plateaus of 20:4ω6 in liver (fig. 1) and heart, of 20:3ω9 in heart and erythrocytes and of 22:5ω6 in plasma and erythrocytes were reached. On the basis of these observations, it appears that the upper limit for the intake of linoleate for the young female rat is 1.2% of calories or approximately 50 mg per day. Fatty acid content of liver lipids of male rats did not show plateaus nor downward trends even at high levels of linoleate intake (11–13). Hence no statement can be made about optimal range of intake of linoleate by male rats based upon these biochemical parameters. However, high intakes of EFA were observed to induce symptoms like those of EFA deficiency and to suppress the weight gain of male rats (11), suggesting that there may be an optimal range of EFA intake for males.

Linolenate-fed rats. In female livers, the levels of 20:5ω3 and 22:6ω3 were two to five times greater than in males at comparable intakes of 18:3ω3. In both sexes the level of 22:6ω3 increased much more sharply at low levels of supplement than did any of the other ω3 fatty acids and reached a plateau. When linoleate was fed, 20:4ω6 behaved in a comparable manner. When supplements exceeded the level of 1% of calories in females and the 3.6% level of calories in males, there was a decrease in concentration of 22:6ω3 in liver lipids. In heart lipid, when dietary linolenate was in excess of 0.5% of calories for females and 1.4% of calories for males, there was no further increase in concentration of 22:6ω3 in females and a decrease in males. In the erythrocytes, the level of 22:6ω3 decreased when dietary linolenate was in excess of 1.4% of calories for male rats and 0.22% of calories for females.

The acids 18:1ω9 and 20:3ω9 decreased when dietary linolenate was fed in increasing doses. Concentration of 20:3ω9 was higher in female than in male tissues and differences were most extreme in the liver. Concentrations of the 18:1ω9 were higher in male tissues, particularly in liver, and the greatest response to diet occurred in liver. The level of 16:1ω7 decreased as dietary linolenate was increased only in the livers of female rats. No consistent trends with diet were apparent in heart or erythrocytes of either sex, or in male livers.

The levels of 16:0 and 18:0 in the livers of females increased slightly with increase in dietary 18:3ω3. Levels of 16:0 were higher and 18:0 lower in male than in female livers. Total saturated fatty acid content was higher in male than in female livers. Members of the linoleate family decreased in quantity in liver lipids of female rats as level of dietary 18:3ω3 increased, in agreement with findings for male rats (11). In liver, as well as in heart, females had the higher concentrations of 18:2ω6 at comparable intakes of 18:3ω3.

Linolenate requirement. In EFA deficiency, oleate metabolism is dominant and 20:3ω9 accumulates in tissue lipids, but when linolenate is fed, 20:5ω3 and 22:6ω3 are synthesized and replace 20:3ω9 in tissue lipids. As the dietary intake of 18:3ω3 increases, the ratio 20:3ω9/20:5ω3 decreases (table 3) and does not change sig-

TABLE 3

Triene-to-pentaene ratios of tissues of rats fed ethyl linolenate

Linolenate		Tissues						
		Liver		Heart		Erythrocytes		Plasma
Male	Female	Male	Female	Male	Female	Male	Female	Female
% of calories								
None	none	65.4	26.1	88.6	21.6	34.3	17.4	23.7
0.01	0.01	76.1	34.4	37.7	24.6	—	45.6	—
0.02	0.02	30.3	42.6	52.7	17.9	—	48.4	—
0.04	0.05	16.2	26.6	61.0	19.7	—	12.1	—
0.08	0.11	5.54	11.1	22.9	11.5	—	7.7	—
0.32	0.22	2.70	4.12	7.1	4.98	—	2.10	2.08
0.61	0.54	1.06	1.09	2.40	1.90	0.60	0.60	0.65
0.77	1.00	0.69	0.47	1.85	1.13	0.44	0.23	0.18
1.42	1.98	0.25	0.25	0.64	0.41	0.21	0.10	0.05
2.56	—	0.09	—	0.20	—	0.07	—	—
3.56	3.91	0.06	0.06	0.15	0.15	—	0.03	0.02
4.14	—	0.04	—	0.14	—	0.14	—	—
9.42	—	0.02	—	0.12	—	0.11	—	—

nificantly below the value of 0.4. The value of 0.4 for the triene-to-pentaene ratio occurred in erythrocytes and plasma at 0.8% of calories of dietary linolenate in liver at 1.0% of calories, and in heart at 2.0% of calories. These intake levels are the same for males and for females and indicate a possible minimal nutrient requirement for linolenic acid.

Although 18:3ω3 did not alleviate dermal symptoms of fat deficiency in female rats, it enhanced weight gain and food efficiency when no linoleate was added to the diet. Therefore, it must perform some needed biological function. Linolenate supplements increase tissue lipid concentrations of 20:5ω3, 22:5ω3 and 22:6ω3. The minimal nutrient requirement of linolenate for the synthesis of adequate amounts of these components of tissue lipids can be deduced from the shapes of their dose-response curves. The minimal nutrient requirement values were calculated from the dose-response curves relating dietary 18:3ω3 to heart and liver 20:3ω9, 20:5ω3 and 22:6ω3 by the method of Caster et al. (16). For suppression of 20:3ω9 approximately 0.5% of calories of 18:3ω3 was required for both males and females and for both heart and liver. For accumulation of 22:6ω3 in liver, 0.2 and 0.14% of calories were required for males and females, respectively. For accumulation of 20:5ω3 in heart 1.0 and 1.7% of calories were required by male and female, respectively, whereas for liver, comparable values were 4.1 and 1.4% of calories, respectively. Similar treatment of weight gain data yielded minimal nutrient requirement values of 0.3 and 1.0% of calories for males and females, respectively. The comparable value deduced from food efficiency data for males was 0.9. These various attempts to compute linolenate requirement yielded somewhat different values which, however, were in the same order of magnitude as those similarly deduced for linoleate requirement. The accumulation of 22:6ω3 to optimal levels occurred at a lower intake of 18:3ω3 than the accumulation of 20:5ω3. The latter represents the buildup of an intermediate, and therefore we consider that of the above data, the suppression of 20:3ω9 and the accumulation of 22:6ω3 represent more likely the requirement for linolenate. Both of these are met by 0.5% of calories in both males and females.

The data presented indicate that metabolic pathways of fatty acid metabolism are the same in the tissues examined here for both male and female rats. Regardless of dietary conditions, however, tissues from female rats consistently had the higher concentrations of 20:4ω6 and 22:5ω6. According to Ostwald et al. (17) this greater efficiency of females to convert 18:2ω6 to 20:4ω6 is a true sex difference and the result of a gonadal hormone effect. They found that EFA-deficient female rats and estrogen-treated, male castrate rats main-

tained higher concentrations of 18:2ω6 in adipose tissue and had higher concentrations of 20:4ω6 in liver and plasma phospholipids than male rats, or testosterone-treated castrates.

The reversal in concentrations of 18:0 and 16:0 of males and females in heart and liver lipid is another true sex effect because diet did not affect it. Similar observations have been reported for plasma and erythrocyte phospholipids (18). Other observed differences which may be classified as true sex effects include the higher levels of 20:3ω9 in tissues of females in deficiency and on low levels of supplement and the greater concentrations of 16:1 and 18:1 in male tissues, as well as their higher triene-to-tetraene ratios at equal intakes of 18:2ω6. The greater proficiency of females in producing the more highly unsaturated metabolites of linolenate, 20:5ω3, 22:5ω3 and 22:6ω3 is probably a hormonal effect also.

Sex differences observed in these experiments are summarized most descriptively by calculation of the number of double bonds per fatty acid molecule. The values for this double bond index for livers and hearts of fat-deficient rats were 0.81 and 0.95 for males and 1.30 and 1.27 for females, respectively. Values for livers and hearts of rats fed 2% of calories of 18:2ω6 were: for males, 0.99 and 1.08 and for females, 1.47 and 1.60, respectively. The number of double bonds per fatty acid molecule for livers and hearts of rats fed 2% of calories of 18:3ω3 were 1.11 and 1.04 for males and 1.79 and 1.47 for females. In each case the tissues of female rats were 1.3 to 1.6 times more unsaturated than those of male rats.

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Derivatives and Analogs of Cysteine and Selected Sulfhydryl Compounds in Nutritional Muscular Dystrophy in Chicks[1]

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ABSTRACT The effects of derivatives and analogs of cysteine and selected sulfhydryl compounds on nutritional muscular dystrophy were studied, using chicks deficient in vitamin E and cystine. The results confirmed earlier indications that mercaptoethylamine is slightly protective against this disorder. Lipoic acid was shown to have a protective effect in a diet marginally deficient but not in a diet severely deficient in sulfur amino acids. Isethionic acid and BAL had no effects on the dystrophy. Of the S-substituted cysteines, S-benzylcysteine accentuated the dystrophy by antagonizing cystine, and did not counteract the effectiveness of vitamin E. S-Carbamylcysteine prevented dystrophy and severely inhibited growth; these effects may have been due to decarbamylation of the compound. S-Methylcysteine and S-ethylcysteine had no effect on dystrophy. The cysteine analog, allylglycine, greatly increased the severity of the dystrophic lesions in the absence but not in the presence of vitamin E. Since the dystrophy-provoking effect of allylglycine was overcome by increased amounts of dietary cystine, these results give additional support to the hypothesis that cysteine is the functional sulfur compound in one of the pathways involved in the prevention of nutritional muscular dystrophy in the chick; vitamin E may be concerned in another pathway.

The effectiveness of cystine in the prevention of nutritional muscular dystrophy in the chicken was first reported by Dam et al. in 1952 (1). Many other sulfur compounds have been examined since for possible effectiveness against this disease. Machlin and Shalkop (2) reported that methionine was fully effective, that sodium sulfate was partially effective, and that thioctic acid (lipoic acid) and taurine were ineffective. Nesheim [3] reported that mercaptoethylamine and thioglycollic acid decreased the severity of muscular dystrophy somewhat, but that 2,3-dimercaptopropanol (BAL) had no effect. Jenkins et al. (3) reported, to the contrary, that mercaptoethylamine and also 3-mercaptopropionic acid were ineffective. Jenkins et al. (4) found that S-methylcysteine had a marginal effect but that S-benzylcysteine had no effect (3).

The present report concerns experiments designed to further investigate the effects of derivatives and analogs of cysteine and selected sulfhydryl compounds on nutritional muscular dystrophy in chicks.

EXPERIMENTAL

Duplicate lots of one-day-old, White Plymouth Rock × Vantress chicks were used for each dietary treatment, with 5 male and 5 female chicks per lot. The chicks were hatched from eggs laid by hens fed a diet low in vitamin E and reared in electrically heated battery brooders with wire-mesh floors. Feed and water were supplied ad libitum. The composition of the basal diet used in the experiments is shown in table 1. Ethoxyquin and sodium selenite were included in the basal diet at levels high enough to prevent encephalomalacia and exudative diathesis, respectively, while allowing the production of muscular dystrophy (5, 6). The severity of the dystrophic lesions was determined at 4 weeks of age by killing the chicks, removing the skin from the breast, and visually scoring the lesions on a 0 to 4 scale.

RESULTS AND DISCUSSION

The results of the first experiment (table 2) show that mercaptoethylamine had a

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[3] Nesheim, M. C. 1959 Studies on the effects of selenium and other factors on vitamin E deficiency in the chick. PhD Thesis, Cornell University.

TABLE 1

Composition of basal diet

	%
Casein [1]	6.00
Isolated soybean protein [2]	7.00
Torula yeast [3]	10.00
Glucose monohydrate [4]	57.10
Cellulose [5]	3.00
Stripped lard [6]	5.00
Vitamin mixture [7]	0.89
Mineral mixture [8]	6.04
Amino acid mixture [9]	4.97
Total	100.0

[1] Sheffield Chemical Company, Norwich, New York.
[2] Assay Protein C-1, Skidmore Enterprises, Cincinnati.
[3] Lake States Yeast, Rhinelander, Wisconsin.
[4] Cerelose, Corn Products Company, Argo, Illinois.
[5] Solka Floc, Brown Company, Berlin, New Hampshire.
[6] Obtained from Distillation Products Industries, Rochester, New York.
[7] The vitamin mixture supplied the following per kg of diet: (in mg) thiamine·HCl, 10; riboflavin, 10; pyridoxine·HCl, 4.5; niacin, 50; folic acid, 4; Ca pantothenate, 20; menadione, 1; choline·Cl, 1490; ethoxyquin, 125; and (in μg) biotin, 200; vitamin B_{12}, 20; and (in IU) vitamin A palmitate, 5600; and vitamin D_3, 4110.
[8] The mineral mixture supplied the following per kg of diet: (in g) $CaHPO_4 \cdot 2H_2O$ 27.22; $CaCO_3$, 13.55; KH_2PO_4, 8.68; NaCl, 7.13; $KHCO_3$, 2.10; and (in mg) $Cu(acetate)_2 \cdot H_2O$, 34; $MnCl_2 \cdot 4H_2O$, 466; $FePO_4 \cdot 4H_2O$, 267; MgO, 850; KI, 2.6; ZnO, 69; $CoCl_2 \cdot 6H_2O$, 1.7; $Na_2MoO_4 \cdot 2H_2O$, 8.3; $Cr_2K_2(SO_4)_4 \cdot 24H_2O$, 48; and Na_2SeO_3, 0.22.
[9] The amino acid mixture supplied the following per kg of diet: (in g) L-arginine·HCl, 10; glycine, 14; L-glutamic acid, 16; L-lysine·HCl, 2.5; L-tyrosine, 1.5; L-tryptophan, 1.2; L-phenylalanine, 0.5; L-leucine, 1.5; L-isoleucine, 0.5; and DL-methionine, 2.0.

slightly beneficial effect on the severity of the dystrophy. However, an equi-sulfur amount of L-cystine completely prevented the disorder. The comparatively small effect of mercaptoethylamine does not suggest a primary role for this compound in the prevention of the disease.

At the concentrations fed, BAL and isethionic acid had no effects on the dystrophy (table 2).

The effectiveness of lipoic acid against muscular dystrophy was reinvestigated in this experiment because of its metabolic importance, and because the previous negative results (2) were obtained by feeding relatively low levels—8 mg or less per kg of diet. In the present experiment, 136 mg of DL-lipoic acid per kg of diet almost completely prevented dystrophy (table 2).

It was reasoned from these results that if lipoic acid plays a primary role in preventing muscular dystrophy, a higher dietary concentration should be completely effective and the level required should be independent of the amount of sulfur amino acids in the diet. The results of the next experiment (table 3) show that in a diet more severely deficient in sulfur amino acids (no added methionine in basal diet), lipoic acid had no effect on muscular dystrophy. However, when added to a diet marginally deficient in sulfur amino acids (0.2% added DL-methionine), lipoic acid again reduced the severity of the dystrophy. Its relative ineffectiveness in the sulfur amino acid deficient diet suggests a sparing, or other nonspecific function of lipoic acid rather than a primary role in preventing the disease.

In the next experiment the effects on muscular dystrophy of various S-substituted derivatives of cysteine were examined (table 4). Contrary to an earlier report (4), S-methylcysteine had no effect on the dystrophy score even though a relatively high dietary level was used. S-Ethylcysteine also had no effect.

Muscular dystrophy was prevented completely and growth was severely inhibited by S-carbamylcysteine. It is possible that these effects were due to decarbamylation of this compound with the release of cysteine which prevented the dystrophy and carbamate ion which inhibited growth. Free carbamate ion spontaneously rearranges in aqueous systems to isocyanate

TABLE 2

Effects of sulfur compounds [1] on muscular dystrophy

Diet	Muscular dystrophy score [2]
Basal diet	0.9[a 3]
+ 0.16% L-cystine	0.0[c]
+ 0.18% MEA·HCl [4]	0.6[b]
+ 0.08% BAL [5]	0.8[a,b]
+ 0.04% isethionic acid	0.9[a]
+ DL-lipoic acid, 136 mg/kg	0.1[c]

[1] All of the sulfur compounds, except cystine, were obtained from Nutritional Biochemicals Corporation, Cleveland. Cystine was from Mann Research Laboratories, New York.
[2] Mean scores for 20 chicks/treatment.
[3] Values followed by different superscripts are significantly different ($P < 0.05$) by Duncan's multiple range test (7).
[4] MEA·HCl indicates 2-mercaptoethylamine hydrochloride.
[5] BAL indicates 2,3-dimercaptopropanol.

TABLE 3

Effects of lipoic acid on muscular dystrophy

Lot no.	Treatments	Muscular dystrophy score [1]	Avg wt, 4 weeks
			g
1	Basal diet 2[2]	1.8[a]	265
2	+ DL-lipoic acid,[3] 136 mg/kg	1.7[a]	267
3	+ DL-lipoic acid, 272 mg/kg	1.9[a]	240
4	+ DL-lipoic acid, 544 mg/kg	2.0[a]	210
5	+ 0.16% cystine	0.0[d]	470
6	As (1) + 0.2% methionine	1.9[a]	477
7	As (6) + DL-lipoic acid, 136 mg/kg	1.6[b]	503
8	As (6) + DL-lipoic acid, 272 mg/kg	1.4[b]	479
9	As (6) + DL-lipoic acid, 544 mg/kg	0.9[c]	418
10	Basal diet 2 + 0.2% methionine + 0.16% cystine	0.0[d]	630

[1] See footnotes 2 and 3, table 2.
[2] Basal diet, table 1, with 0.2% DL-methionine omitted from amino acid mixture.
[3] Lipoic acid obtained from Nutritional Biochemicals Corporation, Cleveland.

TABLE 4

Effects of cysteine derivatives on muscular dystrophy

Treatments [1]	Muscular dystrophy score [2]	Avg wt, 4 weeks
		g
Basal diet	1.1[a]	430[a]
+ S-methylcysteine	1.1[a]	405[ab]
+ S-ethylcysteine	1.0[a]	390[b]
+ S-carbamylcysteine	0.0[b]	261[c]
+ S-benzylcysteine	2.0[c]	390[b]
+ L-cystine	0.0[b]	502[d]

[1] Basal diet, table 1; L-cystine at 0.16%, all other compounds in equi-sulfur concentrations. Cysteine derivatives from Nutritional Biochemicals Corporation, Cleveland.
[2] See footnotes 2 and 3, table 2.

TABLE 5

S-benzylcysteine and muscular dystrophy

Diet [1]	Muscular dystrophy score [2]
Basal diet	1.5[a]
+ vitamin E	0[b]
+ vitamin E + S-benzylcysteine	0[b]
+ cysteine	0[b]
+ cysteine + S-benzylcysteine	1.4[a]

[1] Vitamin E as d-α-tocopheryl acetate at 20 mg/kg, cysteine as L-cysteine at 0.16%, S-benzylcysteine in equi-sulfur concentration.
[2] See footnotes 2 and 3, table 2.

which, if formed, could readily explain the growth depression observed. It appears unlikely that intact S-carbamylcysteine could be related in any way to the primary factor responsible for prevention of dystrophy in view of the severe growth depression it produced. The addition of S-benzylcysteine to the diet increased the severity of the dystrophy. This finding is contrary to that reported earlier by other workers (3), who found no effect of this compound upon dystrophy under their conditions.

In the next experiment, S-benzylcysteine was fed in combination with both cystine and vitamin E to determine whether its dystrophogenic activity was due to an antagonism of cysteine or vitamin E, or both. The results (table 5) show that S-benzylcysteine counteracted the dystrophy-preventing effect of the level of cystine used, but did not interfere with the ability of vitamin E to prevent dystrophy. Preliminary experiments had shown that the levels of cystine and vitamin E fed in this experiment were barely adequate to prevent dystrophy.

The cysteine analog, allylglycine, has been shown to inhibit cysteine metabolism for growth of bacteria (8) and for sulfate production in rat liver mitochondria (9). This compound was fed to chicks to determine its effects on muscular dystrophy. In a preliminary experiment, DL-allylglycine proved to be toxic for the chick: 0.4% caused 75% mortality in 2 weeks, and 0.2% caused 30% mortality by the time the chicks were 3 weeks of age. The results of two further experiments with DL-allylglycine are shown in table 6. In experiment 1, 0.1% allylglycine greatly accentuated the dystrophy as compared with that occurring in chicks receiving only the basal diet. In experiment 2, increasing severity of dys-

TABLE 6

Effect of allylglycine on muscular dystrophy

Lot no.	Treatments [1]	Muscular dystrophy score [2]	Avg wt, 4 weeks
			g
	Experiment 1		
1	Basal diet	0.7^{a}	469
2	+ 0.1% DL-allylglycine	2.5^{b}	426
	Experiment 2		
1	Basal diet	0.8^{bc}	508^{ab}
2	As (1) + vitamin E	0^{a}	521^{abc}
3	As (1) + 0.08% cystine	0.3^{ab}	564^{bcd}
4	As (1) + 0.16% cystine	0.2^{ab}	590^{de}
5	As (1) + 0.32% cystine	0^{a}	596^{e}
6	As (1) + 0.1% DL-allylglycine	1.0^{c}	526^{abcd}
7	As (6) + vitamin E	0.05^{a}	527^{abcd}
8	As (6) + 0.08% cystine	0.7^{bc}	579^{cde}
9	As (6) + 0.16% cystine	0.05^{a}	574^{bcde}
10	As (6) + 0.32% cystine	0^{a}	598^{e}
11	As (1) + 0.2% DL-allylglycine	1.6^{d}	470^{a}
12	As (11) + vitamin E	0.05^{a}	493^{a}
13	As (11) + 0.08% cystine	0.8^{bc}	567^{bcde}
14	As (11) + 0.16% cystine	0.05^{a}	571^{bcde}
15	As (11) + 0.32% cystine	0^{a}	563^{bcd}

[1] Basal diet, table 1. Vitamin E as *d*-α-tocopheryl acetate, 20 mg/kg.
[2] Values followed by same superscript are not significantly different ($P < 0.05$), by Duncan's test (7).

trophy occurred as the dietary level of DL-allylglycine was increased from 0.1% to 0.2%. This appeared to be due to a direct antimetabolite effect upon cysteine metabolism, since the dystrophy-aggravating effects of allylglycine were completely overcome by increasing the levels of dietary cystine.

The effects of DL-allylglycine, like those of S-benzylcysteine (table 5) were overcome by addition of vitamin E to the diet, thereby indicating that the beneficial effect of vitamin E upon nutritional muscular dystrophy in the chick may act directly or indirectly through some function that is independent of the mechanism whereby cystine prevents this disorder.

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Response of Germfree, Conventional, Conventionalized and *E. coli* Monocontaminated Mice to Starvation [1,2]

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ABSTRACT The response of germfree, conventional, conventionalized and *Escherichia coli* monocontaminated mice to starvation was studied. This was undertaken as an extension of some investigations of the effect of microorganisms on the reactions of mice and rats to shock and radiation injury because acute deprivation of food is a common feature of such experiments. Since fasting markedly influences the physiologic and biochemical responses of mice and rats to injury, the results of such experiments reflect the combined effects of injury and food deprivation. Clearly, those factors which condition the animal's response to starvation are of prime importance in evaluating the response to the injury. Conventional and conventionalized mice survived significantly longer than germfree mice when starved. This difference in survival was not due to differences in the rates of loss of body weight by these groups, since the rates of weight loss were the same. The germfree mice died weighing significantly more than the conventional and conventionalized mice. Mice purposefully monocontaminated with a single strain of *E. coli* (sero-group 024) survived longer than germfree mice in one experiment, but this effect was not constant. Again, the rates of weight loss were the same for these groups of mice. The response to starvation of germfree *E. coli* monocontaminatd mice was not altered by parenteral administration of thiamine.

We have been studying the response of germfree and contaminated mice to fasting. These studies were undertaken as an extension of some investigations of shock (1) and radiation injury (2) because acute deprivation of food is a common feature of such experiments. Since fasting markedly influences the physiologic and biochemical responses of animals to injury (3), the results of such experiments reflect the combined effects of injury and food deprivation. Clearly, those factors which condition the animal's response to starvation are of prime importance in evaluating the response to the injury.

EXPERIMENTAL

Lobund [7] mice were used in experiments 1, 2, 4, 5, 6 and Charles River [8] mice in experiment 3. In experiment 1, survival during starvation was compared in 5-week-old, male "germfree" [9] and "conventional" [10] mice. In experiment 2, both male and female germfree and conventional mice, 8 weeks of age, were studied under the same conditions. The germfree and conventional mice used in these 2 experiments were born at the Walter Reed Army Institute of Research in stainless

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[2] A preliminary report of some of these results was made at the annual meeting of the Federation of American Societies for Experimental Biology, April, 1962, Atlantic City, New Jersey.

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[7] A randombred, Swiss albino strain from the Gnotobiotics Laboratory (P. C. Trexler) Lobund Institute, University of Notre Dame, South Bend, Indiana.

[8] These mice were obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts. This randombred strain was started from Swiss albino germfree mice obtained from the Gnotobiotics Laboratory (P. C. Trexler), Lobund Institute, University of Notre Dame, South Bend, Indiana.

[9] "Germfree" mice are mice free of viable bacteria, parasites and fungi as judged by methods published elsewhere (5).

[10] "Conventional" mice differed from ordinary open animal room mice in that the conventional mice were the progeny of open animal room mice moved into and maintained in the same sort of isolators as germfree mice. The conventional mice were born and maintained in isolators throughout their lives.

steel Reyniers series 400 isolators[11] maintained specifically for breeding purposes. At weaning, the animals were transferred into other identical isolator units where they were maintained until completion of the starvation studies.

Male and female animals were reared separately in groups of approximately 5 animals each. The cages used were 20 cm × 30 cm × 13 cm, with 3-mesh galvanized wire tops. Pine wood shavings were used as bedding. After weaning, both germfree and conventional mice received steam sterilized diet L-356 (4) and water ad libitum. Diet L-356 consisted (in percent) of casein, 18; rice flour, 52; corn oil, 4.5; albimini yeast extract, 1.8; dessicated liver, 1.8; minerals, 7; water 9 and a vitamin mixture. Germfree, conventional and open animal room mice eating this diet grow and reproduce well. Temperature control of the isolator units provided a maximal variation between 24° and 27° throughout the experiment with the exception that during autoclaving procedures the temperature rose to 30°–32°, but returned to normal within 2 hours. No autoclaving procedures were carried out after the starvation periods were begun. Routine determinations made on exhaust air indicated a range of 35 to 50% relative humidity. Timers on the lights of the isolators provided 12 hours of illumination and 12 hours of darkness. After the first 3 days of starvation, however, lights were turned on hourly at night to check the condition of the mice. The microbiologic status of germfree animals was determined weekly by procedures previously described (5) except that the phenol red and cooked meat media were not used.

Three days before beginning the starvation regimen in experiments 1 and 2 the mice were transferred into individual 8 cm × 8 cm × 13 cm cubicles made with galvanized metal dividers placed inside the plastic cages. The floor consisted of a false bottom made of 3-mesh galvanized wire screens. During this prestarvation period, the mice were given food and water ad libitum and were weighed each morning, (10 AM to 12 noon). On the morning of the third day, all food and water were removed. Surviving animals were weighed each day throughout the starvation period, care being taken to weigh the animals at the same time each morning. After the third day of starvation, an hourly check of surviving animals was made; the time of death and weight at death were recorded.

The mice used in experiment 3 were born germfree in flexible film plastic isolators (6) at the Charles River Breeding Laboratories, Wilmington, Massachusetts. At weaning, the mice were divided by litter and sex into two paired groups; one group remained germfree, the other was purposefully "conventionalized" at weaning by contaminating them with the cecal contents of open animal room mice. The conventionalized mice were maintained in the same type of isolators as the germfree mice and were fed the same autoclaved diet and water. The mice were transferred to the Albert Einstein College of Medicine when they were 9 weeks old. Male and female mice were reared separately in groups of 4 animals each in plastic cages of the type used in experiments 1 and 2. The food was a commercial ration[12] and was sterilized by steam at the laboratory. Germfree, conventionalized, and open animal room rats grow and reproduce well with this diet. The growth rates of mice eating this ration are similar to those of mice eating diet L-356.

When the mice were 16 weeks old, some were transferred into other plastic isolators and placed in individual cubicles in plastic cages with 3-mesh wire floors and tops just as in experiments 1 and 2. Neither solid food nor water was given after

[11] In the experiments reported here, as in others from our laboratories, we maintained the conventional and conventionalized mice under the same environmental conditions as the germfree and *E. coli* mice with the exception that the first 2 groups had a wide variety of microorganisms. The conventional and *E. coli* mice and their germfree counterparts were kept in stainless steel Reyniers units. Food and other supplies were put into the Reyniers units simultaneously by connecting the isolator autoclaves to the steam lines in parallel. The conventionalized mice and their germfree littermates were housed in flexible film vinyl plastic isolators; entries were made through peracetic acid locks. Air flow, air pressure, humidity, and temperature were kept the same in paired isolators. The food and water (U. S. Coast Guard canned emergency drinking water) of all mice were autoclaved, and the air delivered to the isolators was sterilized by passage through fiberglass mat filters. At intervals of approximately 2 weeks, the closed environments of the conventional and conventionalized isolators were broken by opening the entry lock.

[12] Ralston Purina Company, St. Louis (5010C feed).

transfer and no more peracetic acid entries were made. The mice were weighed when transferred, then each morning, and at death.

Experiment 4 was conducted using procedures similar to those described for experiments 1 and 2 except that survival of the germfree mice following food deprivation was compared with that of mice purposefully monocontaminated with a single strain of *Escherichia coli*.[13] Animals used in this experiment were born under germfree conditions at the Gnotobiotics Laboratory, Lobund Institute, University of Notre Dame, and after weaning were shipped to the Walter Reed Army Institute of Research in specially designed transportation units. Upon arrival, the mice were divided into 2 groups, which were transferred into separate sterile Reyniers units. A 24-hour broth culture of the *E. coli* was then introduced into one of the isolators and 1 to 2 cm^3 of the broth added to each drinking fountain. The starvation periods were begun 9 to 13 weeks later, when the mice were 19 to 23 weeks of age. Husbandry and microbiologic assay procedures identical to those described for experiments 1 and 2 were used. A pure culture of the *E. coli* organism was isolated during each routine microbiological examination of these monocontaminated groups.

Experiment 4 was conducted in four separate trials carried out during a 2-month period. In each trial, groups of 5 to 6 germfree and 5 to 6 *E. coli* monocontaminated mice were starved. Both female and male mice were used, the sex distribution in each individual trial being identical for the 2 groups compared. The conditions of starvation were the same as those described for experiments 1 and 2. In one of the trials, the mice were allowed access to water during starvation; in the other 3 trials, both solid food and water were withdrawn.

In experiment 5 a study was made of the effect of parenteral thiamine administration on the response to starvation of germfree and *E. coli* monocontaminated mice. The mice were received from the Gnotobiotics Laboratory at the University of Notre Dame at 7 to 9 weeks of age and were divided into 2 groups, one of which was immediately contaminated with the same *E. coli* strain used in experiment 4. At 20 to 22 weeks of age, the germfree and *E. coli* monocontaminated groups were each divided into 2 sub-groups of 6 mice. Equal numbers of male and female mice were used in each sub-group. One sub-group of germfree mice and one sub-group of the monocontaminated mice were given 50 μg of thiamine hydrochloride daily, administered intramuscularly in 0.05 ml of distilled water. Injections were made each morning at the time of weighing (10:00 AM to 12 noon) throughout the period of starvation. The other germfree and *E. coli* monocontaminated sub-groups served as controls for the thiamine-injected mice and received 0.05 ml of distilled water intramuscularly each morning at the same times. All injections were made in the thigh muscles with tuberculin-type syringes and 25-gauge needles. Other conditions during starvation were identical to those described for the preceding experiments. At the time of death, the mice were weighed, wrapped in Mylar[14] plastic film and removed from the isolators by means of germicidal traps. This procedure allowed removal of all mice within one hour following death, but eliminated the necessity of repeated steam sterilization of the isolator locks. Following removal, the mice were rewrapped in Mylar plastic film over which was wrapped 2 to 3 layers of aluminum foil. They were then frozen in solid CO_2 where they remained until body composition determinations were made. The techniques used for tissue preparation and analysis were similar to those described by Da Costa et al. (7). Briefly, they consisted of homogenizing the entire mouse, cooling further with liquid nitrogen, in a burr mill which was also cooled with liquid nitrogen. Aliquots of this homogenate were analyzed for water content, for total nitrogen by a micro-Kjeldahl procedure (8) and for total calorie content using an adiabatic bomb calorimeter.

Experiment 6 was conducted in a man-

[13] The *E. coli* strain (serogroup 024) used in these studies was of mouse origin.

[14] Mylar, a polyester (terephthalate) plastic film manufactured by E. I. duPont de Nemours and Company, Inc., Wilmington, Delaware.

ner similar to that described for experiment 5 except that the effect of parenteral thiamine administration on starving germfree mice was compared with its effect on starving conventional mice. Both germfree and conventional mice were born at the Walter Reed Army Institute of Research in stainless steel Reyniers units as in experiments 1 and 2. The mice were transferred into other separate Reyniers units at weaning (approximately 21 days of age). Starvation was begun when the mice were 14 to 15 weeks of age.

The germfree mice were divided into 4 sub-groups of 6 animals each. The sex distribution in each of the groups was approximately the same. One sub-group received 50 μg of thiamine hydrochloride each day as described for experiment 5. A second sub-group served as an injection control receiving 0.05 ml of distilled water. The remaining 2 sub-groups served as uninjected controls, one for the rats injected with thiamine and one for the rats injected with water. The conventional mice were divided into 2 sub-groups, one of which received 0.05 ml of distilled water intramuscularly, while the other served as an uninjected control. All injections were made each morning at the time the mice were weighed. At death, the mice were removed and the tissues analyzed, using the methods described for experiment 5.

Student's *t* test was used for evaluating the statistical significance of the survival and weight loss data. The body composition data were evaluated by analysis of variance.

RESULTS

In experiments 1, 2, and 6 the survival time of the germfree mice during starvation was significantly shorter than that of corresponding conventional mice (tables 1, 2). At a time when all the germfree mice were dead, almost all the conventional mice were still alive. A similar difference was observed between germfree and conventionalized mice (exp. 3, fig. 1). The survival advantage associated with a mixed microbial flora was seen in experiment 4 when a single viable bacterial species, *E. coli* sero-group 024 was present (table 1), but not in experiment 5. In each of the 4 separate trials represented by experiment 4, the *E. coli* monocontaminated mice survived starvation longer than germfree mice. In one of the trials, solid food was withdrawn, but water was allowed ad libitum. As expected, the provision of water was of no survival benefit to either the germfree or monocontaminated groups (fig. 2). In experiment 5, conducted as a single trial, mice monocontaminated with the same *E. coli* strain used in experiment 4 did not survive as long as their germfree counterparts (table 2). The mice in this experiment, but not in experiment 4, received injections of either thiamine or dis-

TABLE 1

Body weight change and survival time during starvation

Exp. no.	Status	No. of animals	Age	Survival time	Body wt	
					Initial	Final
			weeks	*hours*	*g*	*g*
1	Germfree	6	5	92.3 ± 4.4 [1]	21.8 ± 0.30	13.0 ± 0.36
	Conventional	6		137.0 ± 11.4	21.5 ± 0.82	10.7 ± 0.41
	Significance			$P < 0.01$		
2	Germfree	12	8	135.7 ± 12.6	28.8 ± 0.67	16.8 ± 0.27
	Conventional	12		184.7 ± 17.7	28.1 ± 1.40	14.6 ± 0.65
	Significance			$P < 0.01$		
3	Germfree	9	16	110.0 ± 9.1	39.6 ± 1.3	27.1 ± 1.1
	Conventionalized	10		147.1 ± 16.4	34.9 ± 0.72	21.7 ± 0.85
	Significance			$P < 0.01$	< 0.01	
4	Germfree	20	19–23	110.1 ± 11.7	35.5 ± 0.67	22.5 ± 0.59
	E. coli	21		136.2 ± 7.5	35.6 ± 0.39	20.8 ± 0.52
	Significance			$P < 0.01$		

[1] Mean ± SE.

TABLE 2

Changes in body weight and body composition and survival time during starvation

Exp. no.	Status	No. animals	Survival time	Body wt		Fractional wt loss	Body composition of starved mice		
				Initial	Final				
			hours	*g*	*g*	*%*	*% water*	*% protein*	*kcal/g dry tissue*
5	Germfree + thiamine	4	170.7 ± 21.0 [1]	35.2 ± 1.2	19.7 ± 1.0	43.8 ± 2.9	68.0 ± 0.6	23.9 ± 0.6	4.44 ± 0.10
	Germfree + H_2O	6	195.7 ± 18.0	36.0 ± 1.1	19.7 ± 1.1	45.5 ± 2.2	67.1 ± 0.5	23.5 ± 0.6	4.28 ± 0.19
	E. coli + thiamine	6	151.0 ± 11.2	33.5 ± 0.9	20.2 ± 0.10	40.9 ± 2.2	68.7 ± 0.4	23.0 ± 0.5	4.21 ± 0.09
	E. coli + H_2O	6	161.0 ± 26.2	36.0 ± 1.0	20.8 ± 0.8	42.1 ± 2.6	67.3 ± 0.3	23.4 ± 0.6	4.26 ± 0.16
6	Germfree control, a	6	173.8 ± 11.2	33.0 ± 0.5	18.8 ± 0.7	43.3 ± 2.9	67.3 ± 0.6	22.6 ± 0.4	4.12 ± 0.08
	Germfree + thiamine	6	161.3 ± 17.5	31.2 ± 1.2	18.4 ± 1.1	41.7 ± 2.1	68.3 ± 0.5	23.4 ± 0.3	3.92 ± 0.12
	Germfree + H_2O	5	115.4 ± 20.0	30.8 ± 1.4	20.6 ± 1.0	32.5 ± 3.7	67.8 ± 1.1	23.2 ± 0.3	4.40 ± 0.17
	Germfree control, b	6	171.5 ± 31.8	32.3 ± 0.9	19.0 ± 1.2	41.4 ± 2.8	68.2 ± 0.5	22.0 ± 0.3	4.08 ± 0.08
	Conventional + H_2O	6	265.7 ± 38.0	33.2 ± 0.7	16.2 ± 0.9	50.9 ± 3.4	67.7 ± 0.6	23.3 ± 0.5	4.03 ± 0.11
	Conventional control	6	236.5 ± 26.7	32.7 ± 1.1	17.2 ± 1.4	47.5 ± 2.8	68.2 ± 0.7	22.7 ± 0.6	3.51 ± 0.33

[1] Mean ± SE.

tilled water during the starvation period, procedures not used in experiment 4.

The rate at which body weight was lost during starvation was not related to survival time or the presence or absence of viable bacteria (fig. 3). In each of the 6 experiments, a direct correlation was observed between survival time and weight loss at death (fig. 4 and table 2). Mice having the shortest survival time (which, with the exception of experiment 5, was the germfree group) had lost significantly less body weight by the time they died than mice which lived longer. Survival time in these experiments was similar for male and female mice.

In experiments 5 and 6 an attempt was made to prolong survival during starvation by parenteral thiamine administration. Our experiments offer no clear evidence that thiamine administration was of benefit to germfree or *E. coli* monocontaminated mice. In experiment 6, the germfree mice injected with thiamine lived somewhat longer than their water-injected controls, but this difference was not statistically significant. The survival time of the thiamine-injected germfree mice was no different from that of the uninjected germfree mice.

The body composition data obtained in experiments 5 and 6 (table 2) were evaluated by analysis of variance. At death, the composition of germfree mice did not differ significantly in percentage of body water, percentage of body protein, or concentration of calories from that of the *E. coli* or conventional mice.

We observed that diarrhea, as indicated by matting of fecal material on the tail and perineal area, developed a few days before death from starvation in the majority of mice. The incidence of diarrhea appeared to be approximately the same among the germfree, *E. coli* monocontaminated, conventionalized, and conventional mice, although diarrhea may have developed slightly earlier and may have been moderately more severe in some of the germfree mice.

We also noticed that many of the mice became extremely active within 2 or 3 days after starvation began. This hyperactivity continued until just before death when the

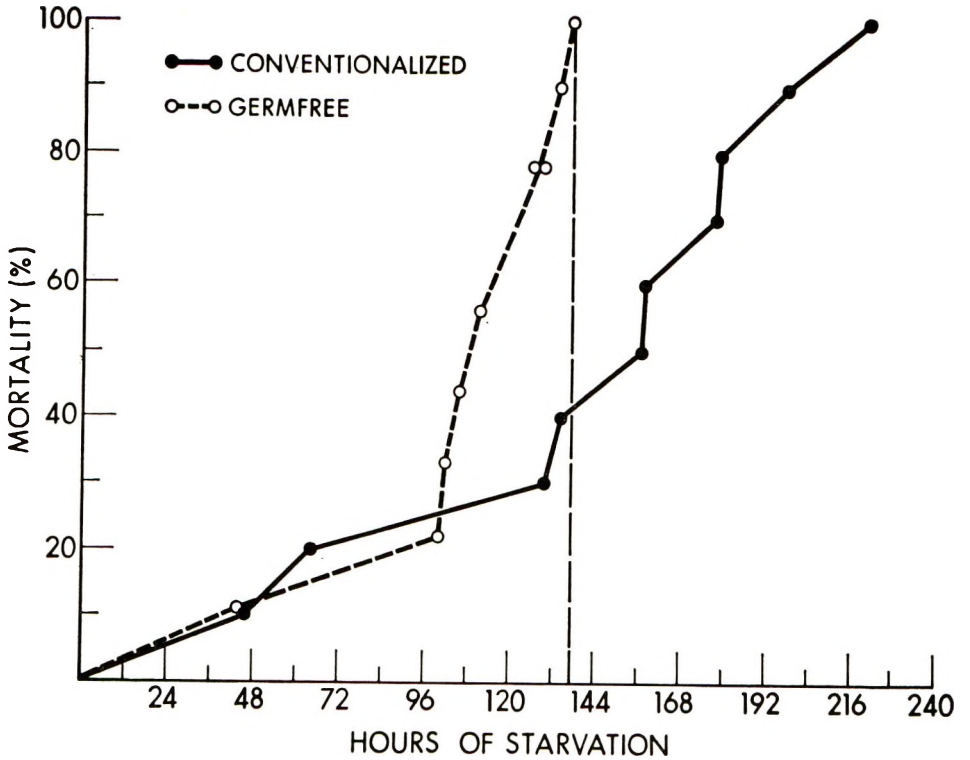

Fig. 1 Starvation of germfree and conventionalized mice: cumulative mortality (exp. 3).

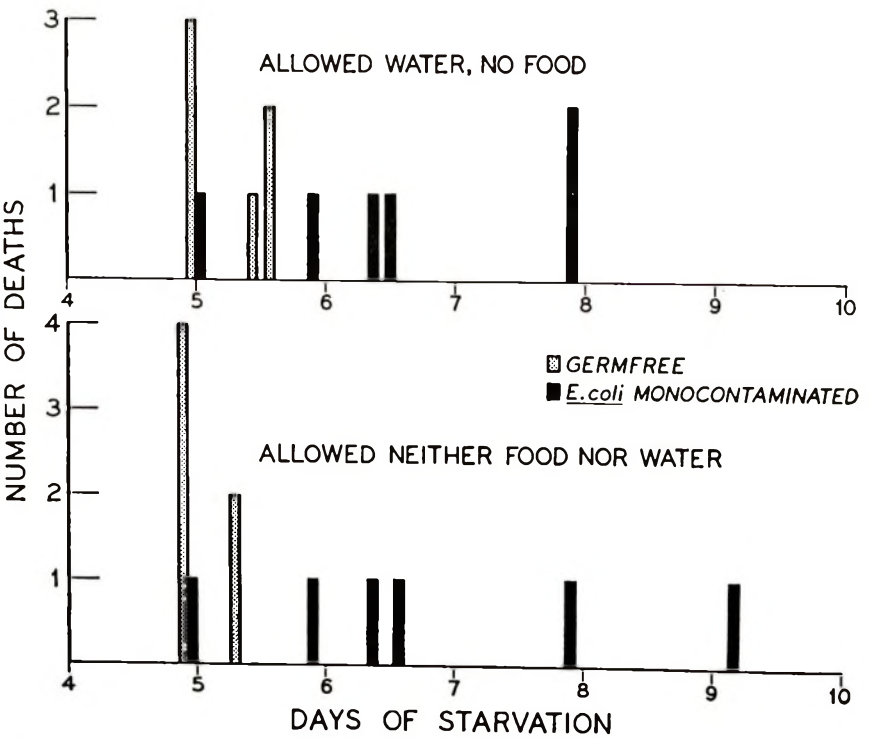

Fig. 2 Survival of germfree and *E. coli* monocontaminated mice allowed water but no solid food or allowed neither solid food nor water (exp. 4).

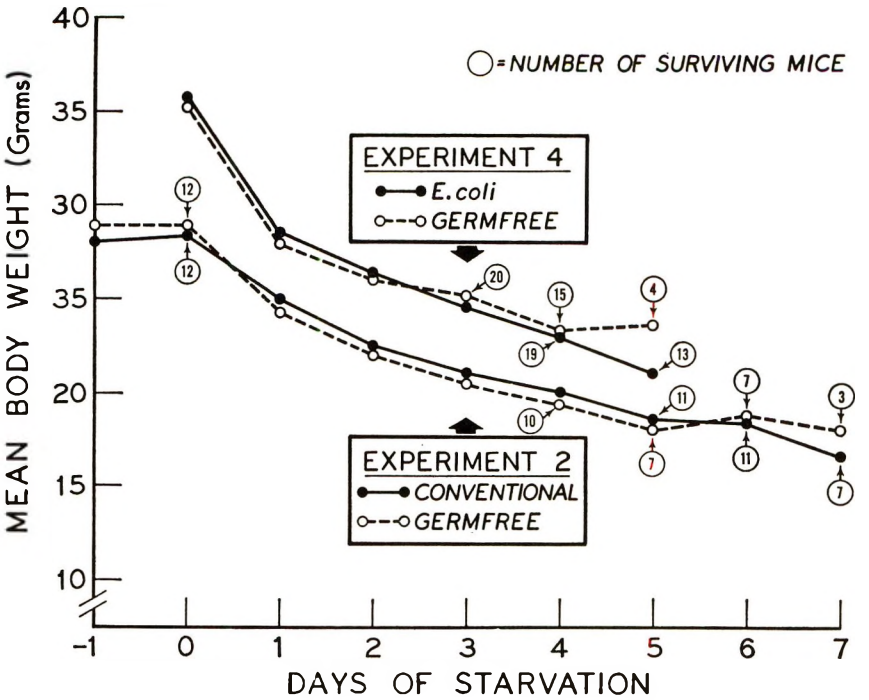

Fig. 3 Change in body weight of *E. coli*, germfree, and conventional mice during starvation; numbers in circles indicate the number of mice surviving.

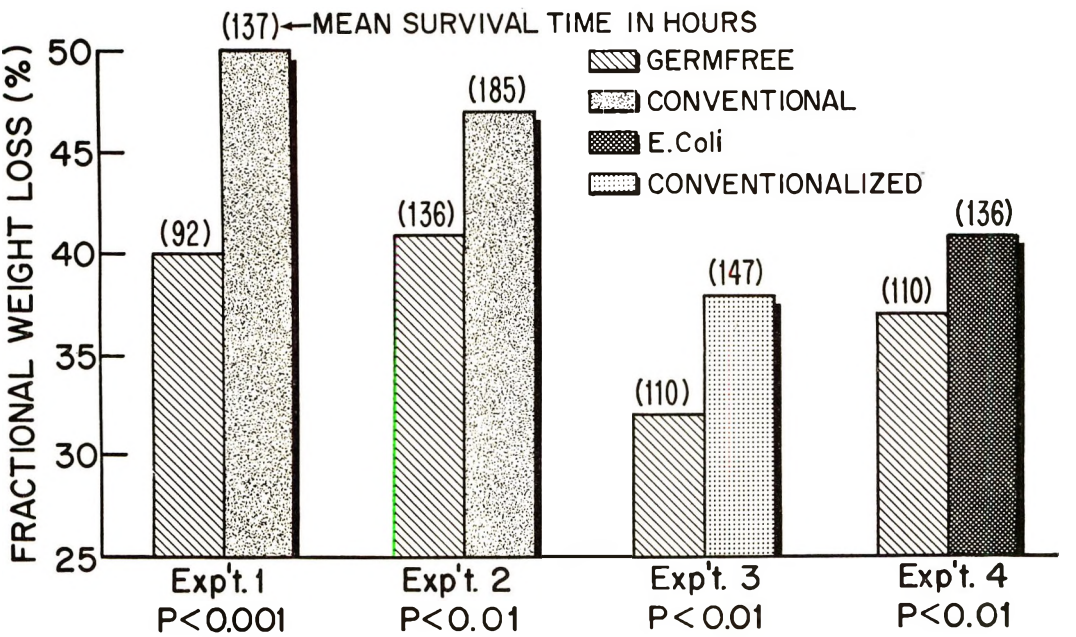

Fig. 4 Relationship between survival during starvation and fractional weight loss of germfree, conventional, *E. coli* monocontaminated, and conventionalized mice.

mice became moribund. We did not observe any difference in the amount of activity among the germfree, *E. coli* monocontaminated, conventionalized, or conventional mice, but no quantitative measurements of activity were made.

DISCUSSION

Experiments 1, 2, 3, and 6 showed clearly that conventional and conventionalized mice survived significantly longer than their germfree counterparts when subjected to starvation. Since the completion of these experiments, we[15] and also Einheber and Carter (9) have made similar observations with rats.

The effect of *E. coli* alone on the survival of mice was variable. The four successive trials of experiment 4 showed that mice monocontaminated with *E. coli* survived starvation significantly longer than germfree mice, but in the single trial of experiment 5, no difference was observed. All mice in experiment 5 received daily intramuscular injections of 0.05 ml of either a thiamine solution or distilled water, while the mice in experiment 4 were not injected. It appears unlikely, however, that the differences between the results of experiments 4 and 5 are due to this, since such injections did not alter survival times in experiment 6.

The differences in response to starvation between germfree mice and those contaminated with microbes may be caused by certain nutritional and metabolic factors which are known to modify the response of ordinary laboratory animals to starvation. For example, the survival of swine (10), rats (11), and mice (12) is related to their total body fat at the start of starvation. In these experiments the body weights of the obese animals at the start of starvation were remarkably greater than those of the non-obese controls. In the study of Smith et al. (12) the mean body weight of the obese mice was 49 g and that of the control group 25 g. Mean body weights at death, however, were similar for both groups, 15.9 g and 15.7 g, respectively. Mean survival time during starvation for the obese group was 21.1 days, and for the controls 6.1 days.

In our studies, no differences existed in initial body weights between germfree mice and their conventional or *E. coli* monocontaminated counterparts with the exception of the lesser weight of the *E. coli* thiamine-injected group in experiment 5. In experiment 3, the conventionalized mice weighed less than their germfree littermates. The differences in survival between these groups therefore cannot be explained on the basis of differences in initial body weight. The survival differences cannot be explained by differential rates of weight loss either, since these rates were the same. The conventional, conventionalized, and the *E. coli* monocontaminated mice of experiment 4, died weighing significantly less than their germfree counterparts. The percentage of body water, the percentage of protein, and the body calorie concentration at death were similar for the germfree mice and for the mice contaminated with either *E. coli* alone or with a mixed microflora (exps. 5 and 6). Since the germfree mice usually died weighing more than contaminated mice, the germfree mice must have died with more total calories remaining.

Although the body weights of the germfree, conventional, and *E. coli* monocontaminated mice were generally the same at the start of the starvation, their body compositions may have differed. There is little published information comparing the body composition of mice such as these. We, therefore, analyzed several groups of germfree, *E. coli* monocontaminated, and conventional mice in other experiments (13). The mice used for these latter tissue analyses were of the same strain and received the same diet as the mice in the starvation studies. We found only slight differences in the percentages of body protein, fat, calories, and water between germfree and conventional mice. The gastrointestinal tracts of the germfree mice were larger than those of the conventional mice, principally because of the enlarged cecums and their contents in the germfree. The cecums of the *E. coli* monocontaminated mice were not significantly different in size from those of the germfree mice. The percentage of body protein was similar in germfree and *E. coli* monocontaminated

[15] Unpublished data, S. M. Levenson and D. Kan.

mice. In 2 experiments, the percentage of body fat of the germfree male mice was significantly greater than that of the *E. coli* monocontaminated male mice, but in the third experiment, no such difference was observed. Tissues of female mice were available for analysis in 2 experiments, and no differences were observed in body fat between the germfree and *E. coli* monocontaminated female mice. The body fat of the females of all groups of mice was, as expected, significantly greater than that of their respective male counterparts.

Differences in overall energy metabolism may have been the basis for the differences in the response to starvation which we observed between the germfree and microbially contaminated mice. However, no information is available regarding such parameters of energy metabolism as oxygen consumption, carbon dioxide production and heat exchange for germfree, conventionalized, and *E. coli* monocontaminated mice during prolonged starvation. The basal metabolism of germfree rats studied 16 hours after eating is significantly lower (15–20%) than that of *E. coli* or conventionalized rats.[16]

Groebbels (14) has observed that the survival time of mice during starvation is markedly reduced if they have previously been fed a diet deficient in certain of the B-group of vitamins. All the mice in our studies received the same steam-sterilized diet which contains, after sterilization, the known vitamins in amounts ordinarily considered generous. There may have been some slight increase in the amounts of certain vitamins available to the microbially contaminated groups because of synthesis by their intestinal microflora, but it appears to us unlikely that this was physiologically significant under the circumstances of our experiments. Although the synthesis of vitamins by bacteria may be maintained during starvation, the amounts of such vitamins would become progressively less, since the numbers of intestinal bacteria are reduced considerably during starvation (15). Further, when ordinary laboratory rats were subjected to complete starvation, the provision of vitamins without a source of food energy had no survival value.[17] In our study no survival benefit followed the injection of thiamine into the germfree and *E. coli* monocontaminated mice in experiment 5. Thiamine was tried because certain intestinal bacteria synthesize thiamine, and germfree rats become depleted of thiamine faster than open animal rats (as judged by growth) when eating a thiamine-free diet (16). In experiment 6 while germfree mice injected with thiamine did not survive longer than uninjected germfree mice, they did survive longer (but not statistically significantly so) than the groups of germfree mice injected with the very small amounts of distilled water (equivalent to the amounts of water in which the thiamine was dissolved). These latter mice behaved in an unexpected manner since they did not survive as long as the uninjected control mice. We have no explanation for this; it was not a consistent finding, since water-injected germfree mice did not survive a significantly shorter time than the thiamine-injected germfree mice, nor did water injection reduce survival in the *E. coli* monocontaminated or conventional groups of experiments 5 and 6.

White et al. (17, 18) have shown that modifications of certain endocrine functions alter the loss of various body tissues during starvation, but little is known about the comparative endocrine functions of germfree, conventional, conventionalized, or *E. coli* monocontaminated mice, fed or starved.

Our observation that diarrhea may have developed earlier and may have been more severe in the germfree mice is, of course, an important point. Further studies to carefully quantitate the incidence and severity of this "hunger diarrhea" are clearly indicated.

Although we did not note any gross difference in activity between the germfree and microbially contaminated mice, quantitative measurements of physical activity are needed to determine whether differences exist.

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Rat Studies on Quality of Protein and Growth-inhibiting Action of Alkaloids of Lupine (*Lupinus termis*)[1]

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ABSTRACT Lupine (*Lupinus termis*) seeds are commonly consumed in the Middle East. The high protein content (35–40%) of the seeds makes them an inexpensive source of dietary protein. Before consumption the untreated bitter seeds are boiled, then soaked in water for several days which removes the bitter taste and the endogenous alkaloid substances in the seeds. This investigation was conducted to evaluate the nutritive quality of the lupine seed protein and the nutritional effects of consuming the alkaloid fraction, by rats. Amino acid analysis showed that, in a 10% lupine protein diet, methionine, lysine and threonine contributed less than half the requirements of the rat for each. Rats fed diets containing 10% protein from untreated lupine seeds failed to grow. Also when an extract of lupine containing the alkaloid fraction was added to a casein diet fed to rats, it suppressed growth severely. Growth of rats fed untreated lupine seeds was improved, however, by removing the alkaloid fraction from the seeds, by increasing the level of dietary protein, and by supplementing the diets with the limiting amino acids. It was concluded that poor growth observed in rats fed untreated lupine diets resulted from both the presence of alkaloid substances and the poor quality of dietary protein. If lupine is to be considered a source of food protein for humans, these 2 factors must be considered in the development of methods of processing lupine seeds for consumption.

Lupine (*Lupinus termis*) is a legume commonly grown in the Middle East. For the most part, the plant is grown as a green manure crop on poor soils; however, the seeds are consumed to a certain extent after being boiled and soaked in water for several days to remove the bitter flavor. This bitter taste of the unprocessed seeds appears to be associated with the presence of certain alkaloids, the chemical nature of which has been reported (1, 2). In addition, there are several reports concerning the pathological effects of these apparently "toxic" materials on experimental and domestic animals (3–8).

The evaluation of legume proteins in food mixtures has been reviewed by Aykroyd and Doughty (9) and it is well-known that the presence in edible legumes of "toxic" substances, such as trypsin inhibitor and hemagglutinins, may affect seriously the nutritional quality of these foods (10). However, little is known concerning the nutritive value of lupine, especially in relation to the alkaloid content. Such information would be of value, especially in the Middle East where there is a shortage of protein, and where legume protein supplements have been found useful for improving local diets (11, 12). Compared with other foods, lupine represents a relatively inexpensive source of food protein. Thus, it may be feasible that this legume be exploited as a major protein food in the Middle East.

The present work was conducted to investigate the nutritive value of lupine from the aspects of protein quality and the presence of inherent growth-depressing factors.

MATERIALS AND METHODS

The raw (untreated) lupine seeds were debittered by soaking in tap water overnight after which they were boiled for 30 minutes to prevent germination. The boiled seeds were then held for 4 days in either running tap water or 4% NaCl solution, after which no bitter flavor was detectable. In the latter soaking treatment the salt solution was changed 3 times daily. The processed seeds were dried at 80° in a forced-draft oven and ground to a fine

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powder (100-mesh) for chemical analyses and for incorporation into animal diets.

Total alkaloids were determined on ground samples by extraction with 95% ethanol according to the method of Gordon and Henderson (13). The AOAC (14) macro-Kjeldahl method was used to determine total nitrogen; crude protein was calculated using the factor 6.25. Amino acids were determined microbiologically (15).

For all animal experiments, test groups of 10 weanling, male, albino rats of the Sprague-Dawley strain [2] were used. The animals were housed individually in screen-bottom cages in an air conditioned room held at 21 ± 1° and relative humidity of 55%. Food and water were offered ad libitum; weight gains and food intake were recorded weekly.

The composition of the basal diet fed to rats in all experiments is shown in table 1. Test proteins were added at the desired level at the expense of cornstarch.

TABLE 1

Composition of basal diet

	g/kg
Cornstarch	800
Nonnutritive cellulose [1]	50
Corn oil [2]	100
Vitamin mixture [3]	10
Mineral mixture [4]	40

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Mazola, Corn Products Company, Argo, Illinois (in glass containers).

[3] Each kilogram of mixture contained the following vitamins, triturated in dextrose: (in grams) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; riboflavin, 1.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in micrograms) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35 (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation).

[4] Contained the following: (g/100 g) ammonium alum, 0.009; Ca biphosphate, 11.28; Ca carbonate, 6.86; Ca citrate, 30.83; cupric sulfate, 0.008; ferric ammonium citrate, 1.526; Mg carbonate, 3.52; Mg sulfate, anhydrous, 3.83; Mn sulfate, 0.02; K chloride, 12.47; K iodide, 0.004; K phosphate, dibasic, 21.88; Na chloride, 7.71; Na fluoride, 0.05 (mineral mixture USP XIV).

Protein efficiency ratio (PER) was determined according to the procedure described by Chapman et al. (16); weanling rats were allocated at random to the test diets containing 10% protein. The possible toxicity of lupine was tested by the method suggested by Campbell [3] in which the growth of animals fed the test food at 10 and 20% protein levels was compared with that of animals fed similar levels of protein from casein.

RESULTS AND DISCUSSION

Data on the protein and alkaloid content of raw (untreated) and debittered lupine seeds are shown in table 2. The protein content of untreated seeds is relatively high, ranging between 35 and 40% by weight. As shown in table 2, the lupine seeds analyzed contained more than 2% by weight of crude alkaloid; it is known that this material is composed mainly of DL-lupanine.[4] After the seeds were boiled and soaked in tap water, the crude alkaloid content was only 0.08%; also the NaCl treatment appeared to be equally effective for removing the alkaloids. The treated seeds were essentially free of the alkaloids and no longer had a bitter flavor.

TABLE 2

Protein and crude alkaloid [1] content of lupine seeds

Treatment of seeds	Protein content	Total crude alkaloid content
	%	%
Untreated	35–40	2.0–2.3
Tap water [2]	39.2	0.080
4% NaCl [3]	38.7	0.077

[1] The alcohol extract (air-dried basis).
[2] Debittered by boiling and washing with water.
[3] Debittered by boiling in water and several washings with 4% NaCl solution.

To study the nutritional effects of feeding the alkaloid fraction, groups of rats were fed casein diets to which were added various levels of the crude alkaloid, obtained by alcohol extraction of raw lupine seeds. The results of this experiment (table 3) show that, even at the lowest level fed, the alkaloid extract had a severe depressing effect on weight gain, food consumption and on the PER values of the diets. From these results, it is apparent that the water-soluble, bitter alkaloid fraction in raw

[2] Obtained from Animal Suppliers (London) Ltd., London.

[3] Campbell, J.A. 1961 A critical appraisal of methods for evaluation of protein in foods. Nutrition Document R. 10 add. 37 WHO/FAO/UNICEF-PAG, United Nations, New York.

[4] Unpublished data, C. Abu Chaar, American University of Beirut, 1966.

TABLE 3

Effect of the addition of crude alkaloid extract on the growth of rats fed a casein diet containing 10% casein [1]

Level of crude extract added	Body wt change, 28 days	Food intake, 28 days	PER,[2] 28 days
%	g	g	
—	107.7	327	3.30 ± 0.22 [3]
0.3 [4]	21.0	170	1.24 ± 0.32
0.6 [4]	19.0	168	1.13 ± 0.20
0.8 [4]	14.0	171	0.82 ± 0.40

[1] Ten male weanling rats/group.
[2] Protein efficiency ratio.
[3] Mean ± SE.
[4] Level of alkaloid in diets 2, 3 and 4 are equivalent to the amount present in diets containing 5, 10 and 13% protein, respectively, in the form of raw lupine.

lupine depressed growth and, presumably, the reduction in food intake was due to the presence of this material.

To investigate whether this crude alkaloid fraction acts as a growth depressant by exhibiting a toxic effect, diets containing 10% and 20% protein from untreated lupine, debittered lupine and casein were fed to groups of rats for 4 weeks. The results of this experiment are shown in figure 1. The weight gain values at 10% protein show clearly that lupine protein is of poor quality, even after debittering. Moreover, the slopes of the curves for the lupine diets, which are an indication of the increase in weight gain obtained with an increase in dietary protein, are lower than for casein. According to the interpretations of Campbell [5] these results suggest that the lupine diets exhibited a "toxic" effect.

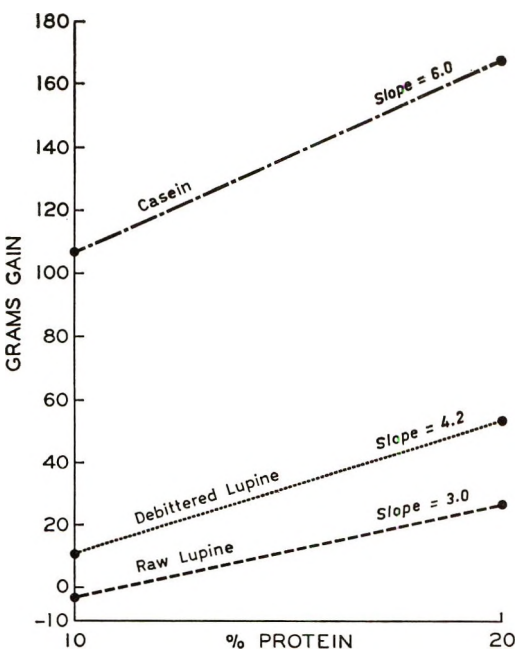

Fig. 1 Difference in 28-day gains of groups of rats fed casein and lupine diets containing 10 and 20% protein (10 rats/group).

As an evaluation of the protein quality of lupine seeds, the content of indispensable amino acids in debittered seeds was determined; these data and the relationship of each amino acid level to the requirement of the growing rat are shown in table 4. Previous experience in this laboratory has shown that the debittering process had no effect on the amino acid content of lupine seeds. According to the data in table 4, all the amino acids listed are limiting to the growth of rats in a diet containing 10% protein from lupine. However, it is clear that the sulfur amino acids, lysine, and threonine are severely limiting to growth, each providing less than half the requirement of the rat.

To investigate further the amino acid deficit in lupine and the effect of increasing the dietary protein level, an experiment was performed in which rats were fed untreated and debittered lupine diets at different protein levels supplemented with various amino acids. The results, presented in table 5, show that the addition of the most limiting amino acids (methionine, lysine and threonine) to a raw lupine diet improved weight gains and PER values significantly. Moreover, further significant improvement in growth and PER resulted from additional supplementation with the

[5] See footnote 3.

TABLE 4

Essential amino acid content of lupine seeds[1] and their contribution in a 10% protein diet

Amino acid [2]	Amino acid content	Requirement of growing rat [3]	Contribution of lupine diet [4]	% of requirement supplied
	g/100 g	*g/kg diet*	*g/kg diet*	*%*
Lysine	1.60	9.0	4.4	49
Threonine	0.92	5.1	2.5	49
Methionine + cystine	0.78	4.9	2.1	43
Phenylalanine + tyrosine	2.75	7.2	6.9	96
Leucine	2.25	6.9	6.1	88
Isoleucine	1.84	5.5	5.0	91
Valine	1.52	5.6	4.1	73
Tryptophan	0.65	1.1	0.8	71

[1] Protein content of debittered lupine seeds, 35.9%.
[2] Determined by microbiological assay.
[3] Rao, B. J., J. Nutr., 69: 387, 1959.
[4] Diet containing 10% protein supplied by lupine seeds.

TABLE 5

Effect of amino acid supplementation of lupine protein diets on the growth of weanling rats

Group [1]	Lupine diets	Dietary protein level	Body wt change	PER [2]
		%	g	
1	Lupine, untreated	10	− 3.0 ± 1.51 [3]	− 0.19 ± 0.021
2	Diet 1 + 3 amino acids [4]	10	22.0 ± 2.13	1.08 ± 0.097
3	Diet 1 + 8 amino acids [5]	10	79.1 ± 7.21	
4	Lupine, debittered	10	10.9 ± 4.59	0.69 ± 0.084
5	Diet 4 + 3 amino acids	10	59.0 ± 4.03	2.23 ± 0.18
6	Diet 4 + 8 amino acids	10	131.5 ± 11.10	
7	Casein	10	107.7 ± 9.31	3.07 ± 0.23
8	Lupine, untreated	20	27.7 ± 2.52	
9	Diet 8 + 8 amino acids	20	114.0 ± 6.37	
10	Lupine, debittered	20	53.2 ± 5.51	
11	Diet 10 + 8 amino acids	20	157.8 ± 7.73	
12	Casein	20	167.1 ± 8.87	

[1] Ten male weanling rats/group; feeding period, 4 weeks.
[2] Protein efficiency ratio.
[3] Mean ± SE.
[4] L-Methionine, 0.25%; L-threonine, 0.2%; L-lysine, 0.2%.
[5] L-Lysine, 0.46%; DL-threonine, 0.26%; L-methionine, 0.28%; L-phenylalanine, 0.03%; L-leucine, 0.08%; L-isoleucine, 0.05%; L-valine, 0.15%; and L-tryptophan, 0.03%.

other 5 limiting amino acids and from increasing the dietary protein level from 10 to 20%. A similar trend in results was obtained with supplementation of the debittered lupine diets. These results (table 5) demonstrate that the protein of untreated lupine seeds is of poor quality. Furthermore, the data show that amino acid supplementation not only improves the quality of the seed protein but also appears to counteract, to some extent, the growth-depressing effect of the alkaloid fraction; there was improvement in growth and PER when untreated lupine diets were supplemented with amino acids.

Similar effects of amino acid supplementation on overcoming the toxicity of raw soybean meal have been reported by others (17, 18). Also, this effect of overcoming the depressing effect of the alkaloids was observed as the level of protein was increased from 10% to 20% in the untreated lupine diets (table 5). Rats fed untreated lupine at the 10% protein level lost weight in 4 weeks, whereas at the 20% level, there was a gain of 27.7 g even though there was a concurrent increase in dietary alkaloid content. The results also show that growth response to debittered lupine diets was greater than that to untreated lupine diets in all comparable treatment, presumably because of the removal of the alkaloid fraction from the seeds.

It can be concluded from the present studies that the growth-depressing effect of diets containing untreated lupine was overcome to varying degrees by 1) debittering the seeds; 2) increasing the dietary protein level; and 3) supplementing the diet with the limiting amino acids. The results show that, of the 3 dietary treatments, amino acid supplementation was the most effective for improving growth of rats fed untreated lupine. Apparently, increasing the dietary protein level did not improve growth to the same extent as amino acid supplementation, probably because of the concomitant increase in the diet of the growth-depressing factor.

It appears that depressed growth of rats fed diets containing untreated lupine seeds is due in part to the presence of the bitter alkaloid fraction and in part to the poor amino acid pattern of lupine protein. Further investigation is needed to determine whether this growth inhibition results from suppressed food intake directly or from an effect on other physiological processes.

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Potassium Requirement of the Weanling Guinea Pig[1]

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ABSTRACT The potassium requirement of the weanling guinea pig was evaluated over an 8-week period, using a diet based on casein protein and containing by calculation 0.3% sodium and 35 mEq of excess cation/100 g of diet. The potassium requirement, estimated from regression analysis, was 0.46% when the potassium was fed as the acetate and 0.54% when fed as the chloride. When suboptimal levels of potassium were supplied as the chloride, the addition of excess cations in the form of sodium acetate improved the growth rate; but when potassium was supplied as the acetate, regardless of the level, the addition of sodium acetate did not significantly influence the growth rate. Animals fed diets based on soybean protein and 0.5% of DL-methionine grew more rapidly than those fed casein diets, but still 0.4% of potassium supported as rapid growth rate as 1.6%. It may be concluded that excess cations are essential in the guinea pig's diet and that the potassium requirement does not exceed 0.5% when the cation level is adequate.

Although the guinea pig is an important laboratory animal, few quantitative studies have been made of its mineral requirements. This species is purported to have an unusually high requirement for potassium, and it is common to supply about 1.4% of potassium in purified diets (1). However, the requirement has not been accurately determined. In early studies Booth et al. (2) found that purified diets did not promote as good growth rate as diets formulated from natural feedstuffs. In a series of experiments it was shown that when the ash (4%) prepared from a practical type diet or alfalfa (equivalent to 25% of the diet) served as the source of minerals excellent growth rates were obtained (2). These results reflected the inadequacy of the salts mixture used originally. Later studies involving the addition of 4 levels of potassium and 4 levels of magnesium to a basal diet containing 0.56% of potassium and 0.04% of magnesium showed that the addition of 1.0% of potassium and 0.3% of magnesium supported the maximal growth rate (3).

It has been reported that sodium in excess of requirement reduces the level of potassium required by the young rat (4). It is not known whether excess sodium will spare the potassium requirement of the guinea pig but it is clear that this species does not tolerate an acid diet (5). Possibly an excess of either sodium or potassium may be beneficial to the guinea pig, as in the case of the rabbit (6), simply by improving the acid-base balance of the diet.

The present study was designed to determine the potassium requirement of guinea pigs fed diets with variable cation-anion balance. The results show that the requirement is considerably less than the 1.4% level recommended by the NRC (1).

EXPERIMENTAL

The composition of the basal diets[2] is shown in table 1. The casein and soybean proteins were found by flame photometric analysis to contain 10 ppm and the total basal diets 30 ppm of potassium. By calculation, the basal diet contained 1.0% calcium, 0.8% phosphorus, 0.3% sodium, 0.16% chloride and 0.3% magnesium, giving a total of 86.7 mEq of cations and 51.1 mEq of anions per 100 g of diet. The cation-anion calculations were made according to Shohl (7) and, accordingly, the balance was not changed by the addition of potassium chloride. The levels of potassium acetate added increased the cation excess by 2.5 mEq (0.1% K) to 40 mEq (1.6% K) per 100 g diet. Sodium acetate was

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[2] The authors gratefully acknowledge gifts of vitamins from Merck and Company, Rahway, New Jersey, Lederle Laboratories, Pearl River, New York, Hoffmann-LaRoche, Nutley, New Jersey, Distillation Products Industries, Rochester, New York and Dawe's Laboratory, Chicago, and Aureomycin from American Cyanamid Princeton, New Jersey.

TABLE 1

Composition of basal diets

	Casein	Soybean protein [1]
	%	%
Casein (reprecipitated)	30.0	—
Soybean protein (washed)	—	30.0
DL-Methionine	—	0.5
Cellulose [2]	15.0	15.0
Soybean oil	4.0	4.0
Salts mixture [3]	4.0	4.0
Magnesium oxide	0.5	0.5
Sucrose	46.4	45.9
Choline·Cl	0.1	0.1
Vitamin supplement [4]	+	+
Antibiotics [5]	+	+

[1] Water added just before feeding, to provide a dough-like consistency.

[2] Solka Floc, Brown Company, Berlin, New Hampshire.

[3] The salts mix supplied as percentage of the diet: $CaHPO_4$, 2.06; $CaCO_3$, 0.91; Na_2HPO_4, 0.65; NaCl, 0.26; Fe citrate, 0.037; $MnSO_4 \cdot H_2O$, 0.064; KIO_3, 0.0016; $ZnSO_4$, 0.0145; and $CuSO_4$, 0.0020.

[4] The vitamin supplement provided per 100 g of diet: (in milligrams) thiamine·HCl 1; riboflavin, 1; pyridoxine·HCl, 1; Ca pantothenate, 3; niacin, 5; folic acid, 0.6; biotin, 0.02; cyanocobalamin, 0.003; inositol, 100; ascorbic acid, 200; α-tocopheryl acetate, 2; menadione, 1; and vitamin A palmitate, 2,000 IU and vitamin D_3, 280 IU.

[5] Chlortetracycline·HCl (Aureomycin·HCl) supplied by American Cyanamid, Princeton, New Jersey.

added so as to provide 12.5 mEq (1.02%) or 25 mEq (2.05%) of excess cation.

The casein was prepared from commercial casein by dissolving it in sodium hydroxide (pH 11) at 52° and reprecipitating with hydrochloric acid at pH 4.6. The precipitated protein was washed repeatedly with distilled water by decantation (10 times), drained and finally dried at 65° for 48 hours in a forced-draft oven. The soybean protein was washed with distilled water (8 times) and dried for 36 hours at 65°. The casein diets were supplied ad libitum in the dry meal form and the soybean protein diets in a moist form. Distilled water was available to the animals at all times.

Weanling male guinea pigs weighing 130 to 150 g were caged in groups of four and 8 to 16 animals were fed each of the respective diets. The experimental design for the casein diets involved 8 levels of potassium, 2 sources of potassium (chloride and acetate) and 3 levels of sodium acetate (none. 1.02% and 2.05%). Two levels of potassium acetate were used in the soybean-protein diets. All supplements were added at the expense of sucrose. The experimental period was 8 weeks and the animals were weighed weekly. Observations were made daily and all dead animals were autopsied. The data were analyzed by analysis of variance and regression analysis (8) and the treatment means were compared by a modified Duncan's multiple range test (9).

RESULTS AND DISCUSSION

Potassium deficiency in the casein diet resulted in a high rate of mortality during the first 4 weeks (table 2). At the 0.1% level of potassium there was 100% mortality even with the addition of sodium acetate to supply excess cation. Mortality decreased with increasing potassium levels, from 0.2 to 0.3%, and was zero at the 0.4% level except for one group. Regardless of diet, animals that survived the first 4 weeks usually continued to grow until the fifty-

TABLE 2

Effect of potassium and cation levels on mortality rate and survival time of guinea pigs fed casein diets

Potassium, %	0.1		0.2		0.25		0.3		0.4	
No. of animals	8		8		8		12		12	
Source of cations	Mortality	Avg survival time	Mortality	Avg survival time	Mortality	Avg survival time	Mortality	Avg survival time	Mortality	Avg survival time
	%	*days*	%	*days*	%	*days*	%	*days*	%	*days*
KCl	100	13	75	13	38	16	25	11	0	0
KCl + 1.02% NaAc	100	9	75	13	—	—	25	17	0	0
KCl + 2.05% NaAc	100	12	75	11	—	—	17	19	25	25
KAc	100	9	50	6	50	9	25	16	0	0
KAc + 1.02% NaAc	100	25	62	14	—	—	25	21	0	0
KAc + 2.05% NaAc	100	16	62	14	—	—	17	19	0	0

sixth day when the experiment was terminated. An explanation for the survival of some animals fed the low levels of potassium is not obvious, but probably relates to individual differences. The metabolic requirements of the surviving animals were either lower than those that died or the processes of absorption and excretion were more efficient in the retention of potassium.

Supplementation of the basal diet with 0.2% of potassium as the acetate or with 0.3% or less as the chloride resulted in significantly lower growth rates than when 0.4% of potassium was supplied by either salt (table 3). The further addition of sodium acetate to the diets containing 0.3% potassium as the chloride produced a significant stimulation in growth rates. The effect was not due to an increase in the sodium ion per se since, in general, no stimulation was observed when sodium acetate was added to diets containing potassium as the acetate. Furthermore, regression analysis showed that animals fed 0.2, 0.25, and 0.3% potassium as the acetate, without the addition of sodium acetate, grew significantly more rapidly than those fed the chloride (fig. 1). Both potassium acetate and sodium acetate supplied excess cations. Since the guinea pig is sensitive to an acid diet, that is, one containing an excess of anions (5), it is reasonable to postulate that both potassium and sodium acetate improved growth by augmenting the guinea pig's cation balance. More extensive studies are required to evaluate the magnitude of this effect. The rate of mortality ranged from 17 to 25% for all groups fed 0.3% potassium but the growth rate of survivors was near maximum except when total cation was limiting (table 3). Clearly 0.3% is not adequate and 0.4% is borderline in meeting the potassium requirement of the guinea pig. Some of the animals fed 0.4% were untidy in appearance and there was 25% mortality among one group. From these results it may be concluded that the minimum potassium requirement for the guinea pig fed casein as the source of protein is between 0.4 and 0.8%. Values estimated from figure 1 place the potassium requirement at 0.46% when fed as the acetate and 0.54% when fed as

TABLE 3

Effect of potassium and cation levels on daily gain of weanling guinea pigs

Potassium		Casein								Soybean protein	
		KCl				KAc				KAc	
		Sodium acetate, mEq				Sodium acetate, mEq				Sodium acetate, mEq	
		0	12.5	25		0	12.5	25		0	
%	*mEq*	*g*	*g*	*g*		*g*	*g*	*g*		*g*	
0.1 [1]	2.5					All animals died					
0.2	5.0	2.7 (2) [2]	3.6 ± 0.60 [3] (2)	3.1 ± 0.60 (2)		3.4 ± 0.61 (4)	3.5 ± 0.19 (3)	3.3 ± 0.60 (3)			
0.25	6.2	4.0 ± 0.36 (5)				4.9 ± 0.25 (4)					
0.3 [4]	7.5	3.7 ± 0.36 (9)	5.1 ± 0.32 (9)	5.3 ± 0.24 (10)		5.1 ± 0.24 (9)	5.3 ± 0.16 (9)	5.3 ± 0.12 (10)			
0.4	10.0	4.7 ± 0.29 (12)	5.3 ± 0.30 (12)	5.1 ± 0.32 (9)		4.8 ± 0.25 (12)	5.4 ± 0.42 (12)	5.1 ± 0.15 (12)		6.9 ± 0.17 (16)	
0.8	20.0	5.1 ± 0.30 (12)	5.3 ± 0.14 (12)	5.4 ± 0.17 (12)		5.5 ± 0.17 (12)	5.2 ± 0.42 (12)	5.1 ± 0.25 (12)			
1.2	30.0	5.1 ± 0.25 (12)	5.2 ± 0.20 (12)	5.0 ± 0.23 (12)		5.5 ± 0.29 (12)	5.0 ± 0.32 (12)	5.0 ± 0.14 (12)			
1.6	40.0	5.3 ± 0.23 (12)	5.0 ± 0.18 (8)	5.2 ± 0.13 (8)		5.4 ± 0.35 (12)	5.3 ± 0.24 (8)	5.0 ± 0.19 (8)		7.1 ± 0.21 (16)	

[1] Eight animals were fed the 0.1, 0.2, and 0.25% levels of potassium and there were 12 in most of the other groups.
[2] Number of survivors shown in parentheses.
[3] SE of mean.
[4] Statistically the growth rates at all levels of potassium enclosed by the same line were not significantly different at the 5% level except for the one group fed 0.3% potassium as the chloride.

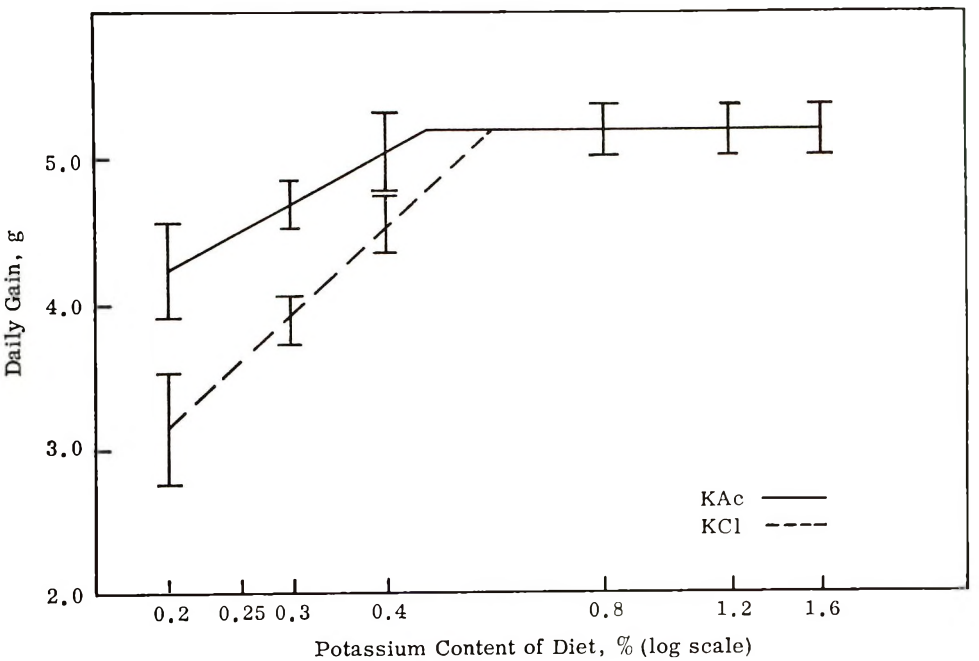

Fig. 1 Graphical representation of the statistical analysis of the growth data of guinea pigs fed potassium acetate and potassium chloride without the addition of sodium acetate. The calculated values and standard errors for the 0.2 to 0.4% potassium levels were computed by regression analysis, while the calculated values and standard errors for the 0.4 to 1.6% potassium levels were computed by pooling the data from both the potassium acetate and potassium chloride treatments since these means were not significantly different.

the chloride. Higher levels of potassium up to 1.6% did not significantly influence the growth rate.

For the guinea pig arginine is the first growth-limiting amino acid in casein (10–12). Because of this limitation a more adequate source of amino acids, soybean protein supplemented with DL-methionine, was used to compare 0.4 and 1.6% potassium. When soybean protein supplemented with 0.5% DL-methionine was substituted for casein the growth rate of animals fed the soybean protein was significantly better than that of animals fed casein diets. The growth rate compares favorably with values reported for a commercial pelleted ration (13), a casein-gum arabic diet (3) and diets containing hay, grain, carrots, rutabagas and fresh grass (14). It is noteworthy that 0.4% of potassium supported the maximal rate of gain when this more adequate amino acid source was fed since no significant difference in growth was observed between 0.4 and 1.6% potassium.

Potassium is not stored in the skeleton to a significant extent as in the case of mineral ions such as magnesium. This is reflected by the fact that the growth rate and survival decreased abruptly as the level of potassium was decreased to the range of one-half to one-third of the minimal requirements (fig. 1 and table 2). In contrast magnesium, which is stored in bone, can be reduced to a quarter or sixth of the minimum level before the survival rate is greatly decreased. There is also a more gradual decrease in growth rate with suboptimal levels of magnesium (15). If excess potassium were stored in the animal body, one would expect the growth response curve to be similar to that of the magnesium.

The NRC recommended level of potassium (1.4%) appears to be in excess of the requirement for guinea pigs fed diets containing 35 mEq or more of excess cations per 100 g of diet.

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Acute Physiological Effects of Feeding Rats Non-urea-adducting Fatty Acids (Urea-Filtrate)[1]

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ABSTRACT Experiments were conducted to further describe the biological response to toxic components of severely heated fats. Much of the toxicity of severely heated food oils has been associated with a non-urea-adducting fatty acid (urea filtrate) fraction. Feeding this fraction on two consecutive days to weanling rats caused inanition, fatty liver, a decline in body temperature and death within 2 to 7 days. In this study the LD_{50} of the fraction, administered orally, was approximately 0.6 ml/100 g/day for 2 days for rats weighing 40 to 50 g; from 60 to 100 g the LD_{50} was approximately 0.9 ml/100 g/day for 2 days. Water consumption had little or no effect upon survival or level of liver fat, whereas forced feeding of non-lipid food elements decreased mortality and prevented the increase of liver lipid. A four- to fivefold elevation in neutral fat accounted for the increased liver fat. No unidentified fatty acids of the urea filtrate were detectable in the liver. The urea filtrate caused a 30% reduction in the conversion of palmitic-1-^{14}C acid to $^{14}CO_2$ in the young rats during a 5.5-hour test period, whereas the oxidation of D-glucose-^{14}C (uniformly labeled) to $^{14}CO_2$ was not affected. These results indicate that one or more of these non-urea-adducting fatty acids appreciably impaired the oxidation of fatty acids in young rats.

In 1961 Friedman et al. (1) reported the results of a nutritional study in rats of a cottonseed oil which had been heated to 225° in the presence of air for about 190 hours. Growth data demonstrated a decreased nutritive value that was not due simply to decreased digestibility. Enlargement of the liver, kidney and thymus was observed at autopsy. The greatest change was an increase in liver weight of more than 27%. The heated oil was separated by distillation and urea-adduction into 4 fractions: unaltered fatty acids, non-adducting fatty acids (urea filtrate), dimeric fatty acids and polymers. The fractions responsible for the observed effects were the urea filtrate fatty acids and the dimers (1, 2).

It is well-documented (3) that heating and oxidation produces toxic materials in a wide variety of fats and oils. The toxicity has been repeatedly related to the peroxides, or subsequent oxidation products, and cyclic compounds. Cyclic fatty acids have minimal effect upon intestinal contents and are well-absorbed which suggests that the toxicity is exerted systemically while the poorly absorbed peroxides exert their major effect upon the cells or contents of the intestinal tract. The urea filtrate fatty acids used for the present studies contain cyclic material and have no detectable peroxide oxygen (4).

This report presents data on the physiological and biochemical abnormalities produced by urea filtrate fatty acids in young rats subjected to the acute toxicity test (4).

Rats of the Osborne-Mendel strain [4] were assigned at random to groups except for 2 experiments (body temperature and the fasting liver lipids), in which littermates were used. Our earlier studies did not indicate any sex-related differences; however, approximately equal numbers of males and females were used. The rats were placed in individual cages; they were observed and weighed daily. Unless otherwise specified, supplements were force-fed by stomach tube at the level of 1 ml/100 g body weight and drinking water was always available. The weanling rat bioassay for

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[1] Part of the data was presented at a meeting of the American Institute of Nutrition at Chicago, 1961.

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urea filtrate toxicity was that described by Firestone et al. (4). The urea filtrate material used was prepared by the procedure of Firestone et al. (4) from a pot-still residue [5] remaining after the commercial distillation of stearic and oleic acids from mixed fats. The urea filtrate used in part of the LD_{50} study was prepared in the same manner from heated cottonseed oil (2). Briefly, the urea filtrate is obtained as follows: a solution of the acids in alcohol (90%) at 50° is brought to saturation with urea; after cooling overnight at room temperature the mixture is filtered (Buchner funnel) and washed with urea saturated 90% alcohol; the resultant filtrate is acidified and extracted to obtain the filtrate fatty acids.

Procedure for LD_{50}. Rats were assembled into three weight ranges: 40 to 50 g, 60 to 70 g, and 90 to 100 g (table 1). Rats in each of these groups were force-fed the urea filtrate at 0.4, 0.6, 0.8 or 1.0 ml/100 g body weight. The filtrate was fed on the first 2 days and the rats were observed through the following 5 days (food [3] and water ad libitum). An additional 40 to 50 g group was fed urea filtrate prepared from heated cottonseed oil (2). Data were obtained from 8 to 23 animals per dose level in each weight range. Regression lines for mortality on dose were calculated for each weight range group and compared statistically.

Body temperature. Six litters of weanling rats, weighing between 30 and 40 g, were divided equally by sex into 5 groups (table 2). They were fasted overnight, then fed the urea filtrate at 0, 0.25, 0.50, 0.75 and 1 ml/100 g body weight. Cottonseed oil was fed to bring the total administered dose to 1 ml/100 g body weight. Temperatures were determined hourly for 8 hours and at 24 hours, with rectal thermistor probes.

[5] Emery Industries, Inc., Cincinnati.
[6] Sheppard, A. J. 1965 Evaluation of four stock diets for liver lipid research on rats. I. Influence of diet on body weight and liver composition. (Diet 4) J. Assoc. Offic. Agr. Chem., 48: 22.

TABLE 1

Relation of body weight of young rats to the urea filtrate toxicity

Group [1]	Wt range	No. deaths/total no. rats [2] Dose, ml/100 g/day for 2 days 0.4	0.6	0.8	1.0
	g				
A	40– 50	5/23 (22)	10/19 (53)	11/15 (73)	13/15 (87)
B	60– 70	0/23 (0)	3/17 (18)	7/22 (32)	13/18 (72)
C	90–100	0/23 (0)	1/19 (5)	6/15 (40)	10/14 (71)
D	40– 50	1/20 (5)	4/16 (25)	10/12 (83)	8/8 (100)

[1] Groups A-C received urea filtrate prepared from the pot-still residue, group D received urea filtrate from heated cottonseed oil. Period of observation was 5 days.
[2] Numbers in parentheses indicate percentage of deaths.

TABLE 2

Effect of urea filtrate upon body temperature of fasting weanling rats [1]

Cottonseed oil	Urea filtrate	Body temperature 3 hr	6 hr	24 hr
ml/100 g	*ml/100 g*	°	°	°
		Control		
1.00	—	36.9 ± 0.25	37.4 ± 0.18	35.7 ± 0.45
		Experimental [2]		
0.75	0.25	−0.4 ± 0.47	−0.4 ± 0.69	−0.4 ± 0.73
0.50	0.50	−1.1 ± 0.34 [3]	−1.5 ± 0.67	−1.9 ± 1.02
0.25	0.75	−1.0 ± 0.43	−1.8 ± 0.66 [3]	−2.1 ± 0.67 [3]
—	1.00	−1.3 ± 0.69	−2.8 ± 0.88 [3]	−4.1 ± 0.70 [4]

[1] Littermates, 6 rats/group, body weight 30 to 40 g.
[2] Temperature change with experimental levels, as difference from the control rat receiving only cottonseed oil, mean ± SE.
[3] Significantly different from control group, $P < 0.05$.
[4] Significantly different from control group, $P < 0.001$.

Liver fat. Observations suggested a strong relationship between survival time and food intake; therefore experiments (table 3) were performed to determine the effect of various food materials on the toxicity of the urea filtrate, including development of fatty livers. In experiment 1, 3 groups of weanling rats (2 males and 2 females/group), weighing approximately 40 g were fasted overnight. The urea filtrate was force-fed to all animals at approximately 9:00 AM on the first 2 days. Groups 1 and 3 were also force-fed daily 3.3 ml of water at approximately 9:00 AM, 1:00 and 5:00 PM. Group 2 was force-fed 3.3 ml of undiluted evaporated milk 3 times/day, on the same schedule as the water for groups 1 and 3. Group 3 received 1.5 ml cottonseed oil/day (9:00 AM) in addition to the water. Surviving animals were killed for autopsy at 7 days. In experiment 2, three groups were force-fed the urea filtrate. Group 5 was force-fed 6 ml of 10% glucose; group 6, 6 ml water; and group 7, 6 ml water plus 0.3 ml fresh cottonseed oil. Group 4 was a fasting control. These rats were killed approximately 18 hours after the urea filtrate was fed and the composite total liver fat was determined.

Two subsequent experiments (table 4) were carried out to determine the effect of urea filtrate on the classes of lipids in the liver. In experiment 1, one group of rats was force-fed cottonseed oil and another group was force-fed urea filtrate at 1 ml/100 g body weight. After 18 hours the livers were removed for lipid analysis. This experiment was repeated (exp. 2) with 10 rats per group. The finely chopped liver tissue was denatured by addition of an approximately equal volume of 95% ethanol and dried in a vacuum oven at 100 to 105°. Total lipid was determined gravimetrically as the petroleum ether-soluble material obtained from a 24-hour Soxhlet extraction with ethyl ether. Total cholesterol was determined in the liver fat by the method of Abell et al. (5) and the free cholesterol according to Sperry and Webb

TABLE 3

Effect of concurrent forced feeding on toxic response to urea filtrate

Exp. no.	Group [1]	Urea filtrate [2]	H_2O	Concurrent feeding	Response	
					Mortality [3]	Liver fat [4,5]
			ml/rat/day	*ml/rat/day*	%	%
1	1	+	10	none	100 (day 4)	—
1	2	+	—	milk, 10	0	—
1	3	+	10	cottonseed oil, 1.5	100 (day 3)	—
2	4	—	ad lib.	cottonseed oil, 0.4	—	4.2
2	5	+	—	10% glucose, 6	—	5.8
2	6	+	6	none	—	8.2
2	7	+	6	cottonseed oil, 0.2	—	9.4

[1] Four rats (2 male and 2 female, approximately 40 g body weight) per group.
[2] Groups 1–3 were force-fed urea filtrate (non-urea-adducting fatty acids) at 1 ml/100 g body weight on first 2 days; groups 5–7 on one feeding only.
[3] Groups 4–7 killed after 18 hours.
[4] Groups 1 and 3 appeared fatty, group 2 appeared normal on seventh day.
[5] Total composite liver fat/group (fresh weight).

TABLE 4

Fasting liver lipids 18 hours after force-feeding urea filtrate [1]

Supplement	No. rats	Exp. no.	Total lipid	Cholesterol		Phospholipid	Neutral [2] lipid
				Free	Total		
				g/100 g liver wet wt			
Cottonseed oil	4	1	4.25 ± 0.81 [3]	—	0.29 ± 0.03	2.82 ± 0.58	1.08
	10	2	4.15 ± 0.40	0.19	0.26 ± 0.01	2.69 ± 0.10	1.16
Urea filtrate	5	1	8.20 ± 1.51	—	0.25 ± 0.02	2.81 ± 0.29	5.10
	10	2	7.76 ± 0.76	0.17	0.22 ± 0.02	2.78 ± 0.11	4.74

[1] Littermate rats, approximately 40 g body weight, were force-fed non-urea-adducting fatty acids (urea filtrate) or cottonseed oil at 1 ml/100 g body weight.
[2] Determined by difference (cholesterol ester as oleate).
[3] Mean ± SE.

(6). Phospholipid (lipid phosphorus × 25) was determined by the acid digestion method of Lowry et al. (7) and the "Elon-molybdate" color reagent (8). Neutral fat was estimated by difference. Gas chromatographic analyses were performed with a Perkin-Elmer Vapor Fractometer and an Aerograph 600 C using diethylene glycol and ethylene glycol-succinate columns. A fatty acid pattern was obtained of the pooled adipose tissue depots (scapular, perirenal, abdominal and testicular) from weanling rats.

Utilization of isotopic glucose or palmitic acid. Weanling male rats (about 40 g), fasted overnight, were intubated with either cottonseed oil or urea filtrate at 1 ml/100 g body weight just before an intraperitoneal injection of 1 ml substrate/100 g body weight. The substrate was either D-glucose-^{14}C, uniformly labeled,[7] 80 mg (4 μCi)/ml, or palmitic-1-^{14}C acid,[8] 10 mg (5 μCi)/ml. Two rats were placed in each metabolic chamber and the expired CO_2 was collected in 150 ml of 3 N NaOH at 0.5, 1, 1.5, 2.5, 3.5, 4.5 and 5.5 hours after injection. Suitable aliquots of the NaOH traps were added to counting vials containing a thixotropic gel [9] and counted for 10 minutes in a Nuclear-Chicago Mark 1 liquid scintillation counter. Correction for quenching was made by the external standard technique. Counting efficiency was approximately 60%.

RESULTS AND DISCUSSION

The susceptibility of weanling and larger, older rats to the toxicity of the urea filtrate is presented in table 1. The regression equations for the 3 groups were as follows: group A (40–50 g), $Y_A = 47.7\ X + 1.352$; group B (60–70 g), $Y_B = 73.5\ X - 1.890$; group C (90–100 g), $Y_C = 102\ X - 4.388$. Because the slopes of these regressions did not differ significantly, the combined slope 60.32 was used for the statistical analyses. The calculated regression equations are as follows: group A, $Y_A = 60.32\ X + 0.357$; group B, $Y_B = 60.32\ X - 0.699$; group C, $Y_C = 60.32\ X - 0.765$ where X = log dose (ml/100 g body weight) and Y = mortality in probits. Group D, which received the heated cottonseed oil urea filtrate, was calculated separately; $Y_D = 88.1\ X - 2.231$. The LD_{50} (ml/100 g body weight/day for 2 days) for each of the groups are as follows: A, 0.589 ± 0.037; B, 0.881 ± 0.053; C, 0.903 ± 0.058; and D, 0.662 ± 0.033. Significant differences ($P < 0.05$) were noted only for the responses between groups A and B, and A and C. The urea filtrate fraction of heated cottonseed oil had a toxicity similar to that of the "by-product" fatty residue (groups A and D). These figures show that the young rat (< 50 g body weight) has a significantly lower tolerance for the toxic material than the heavier rat.

We noted, during this study, that the animals receiving the urea filtrate felt cold to the touch soon after dosing. Data in table 2 verify and quantitate this observation. A rather large significant decline in body temperature resulted from feeding the urea filtrate. A similar but much more variable response was obtained within 1 to 2 hours after intraperitoneal injections of one-fourth the oral dose of urea filtrate.

Early observations had shown that after force-feeding a toxic dose of the urea filtrate the food and water consumption were very greatly reduced. This is in contrast with the increased water consumption generally observed in the chronic studies (3). An experiment was performed to determine whether dehydration alone was responsible for the effect. Weanling rats with access to dry ration [10] survived complete water deprivation for 7 days whereas all 5 rats of a similar group fed urea filtrate succumbed in less than 3 days. Furthermore, observations at autopsy indicated that dehydration, per se, was not the cause of death.

Preliminary experiments indicated that food, taken either before or shortly after dosing with urea filtrate, improved the animal's chance for survival. Table 3 shows the effect of force-feeding food. The addition of cottonseed oil (group 3) in-

[7] New England Nuclear Corporation, Boston.
[8] See footnote 7.
[9] The thixotropic gel was prepared by mixing 50 g CAB-O-SIL (Packard Instrument Company, Downers Grove, Illinois), 100 g naphthalene, 0.3 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 7.0 g 2,5-diphenyloxazole (PPO). Fifteen milliliters were added to 1 ml of sample and 2 ml methanol.
[10] See footnote 6.

TABLE 5

Fasting liver lipid fatty acids 18 hours after force feeding urea filtrate [1]

Fatty acid	Supplement	
	Cottonseed oil	Urea filtrate
	%	%
12:0	1.0	2.5
14:0	1.6	3.6
14:1	0	0.1
16:0	28.2	22.2
16:1	0.5	1.0
18:0	21.9	11.2
18:1	10.7	21.7
18:2	20.3	27.6
20:4	15.8	10.1
Total	100.0	100.0

[1] Combined liver lipids for each supplement group of 10 rats force-fed non-urea-adducting fatty acids (urea filtrate) or cottonseed oil at 1 ml/100 g body weight.

creased the toxicity of urea filtrate and produced death in less than 3 days, but no deaths occurred in the milk-fed group (group 2). The livers of group 2 were not fatty on macroscopic examination, but both groups 1 and 3 did appear fatty. Since milk is a complex mixture, the possible effects of a simple sugar were tested (group 5). The data cannot be evaluated statistically because the values were for composite samples; however, the 29% lower total liver fat in the group fed glucose (600 mg/rat) as compared with the group receiving only urea filtrate would probably be significant (group 5 vs. group 6). The nearly 15% increase in liver fat of group 7, fed cottonseed oil (180 mg/rat), may also be significant. At autopsy, a large portion of the administered dose of urea filtrate was consistently found in the stomach. The amount in the stomachs of the rats of groups 5 and 6 was approximately 60% of the administered dose. This recovered material was tested and was equal in toxicity to the original urea filtrate.

The data in table 4 delineate the classes of lipids in the liver after the urea filtrate was fed. No changes were evident in the cholesterol or phospholipid fractions; hence the increase in total lipid was due to the more than fourfold increase in neutral fat.

The liver lipids from experiment 2 (table 4) were combined by group for gas chromatographic analysis of the fatty acids (table 5). The urea filtrate caused deviations from the fatty acid pattern of the rats fed cottonseed oil. The 18:0 and 18:1 fatty acids were particularly different. From the gas chromatographic pattern of the urea filtrate itself, 41.6% of the total fatty acids had retention times corresponding closely to known fatty acid standards. Oleic acid, present at 35.9%, was the major component. The remaining unidentified fatty acids had retention times principally in the general range 15:0 through 19:0 acids; however, they did not correspond to specific known acids. None of the unidentified components was evident in the liver fat of rats that received the urea filtrate.

TABLE 6

Comparison of adipose tissue fatty acids with liver fatty acid changes after feeding urea filtrate

Fatty acid	Liver fatty acids				Adipose tissue fatty acids
	Treatment		Difference		
	Cottonseed oil	Filtrate			
	mg/g liver [1]	*mg/g liver* [1]	*mg*	%	%
12:0	0.29	1.56	1.27	3.8	3.3
14.0	0.46	2.25	1.79	5.4	3.7
14:1	0	0.06	0.06	0.2	0.8
16:0	8.18	13.86	5.68	17.0	21.8
16:1	0.14	0.62	0.48	1.4	4.1
18:0	6.35	7.00	0.65	2.0	3.5
18:1	3.10	13.55	10.45	31.3	27.8
18:2	5.89	17.22	11.33	33.9	35.0
20:4	4.59	6.30	1.71	5.0	—
Total	29.00	62.42	33.42	100.0	100.0

[1] Calculated from data in tables 4 and 5 assuming that the fatty acids represent 70% of the total liver fat for the cottonseed oil group and 80% for the "filtrate" group (cholesterol (oleate), 42.2% fatty acid; phospholipid (oleate), 70.2%; triolein, 95.7%).

Fatty acid synthesis is minimal during fasting (9); hence the accumulated liver fatty acids of the filtrate-fed group undoubtedly originated principally in adipose tissue. The fatty acids of the depot fat were compared with the excess liver fatty acids accumulated in the filtrate-fed rats. It was assumed that the fatty acids represented 70% of the total liver fat for the cottonseed oil group and 80% for the filtrate group (table 6). From the fatty acid analyses the quantities of each fatty acid present in 1 g of fresh liver was calculated. The difference between the 2 groups, which represented the accumulated lipid due to urea filtrate, had a profile similar to that of the adipose tissue.

The responses due to feeding urea filtrate characterized thus far include 1) accumulation of liver lipid, 2) marked decline in body temperature, 3) death, 4) enhancement of the toxic responses by concomitant fat feeding, and 5) protection by concomitant feeding of carbohydrate or carbohydrate-protein. These responses suggest that the ability of the weanling rat to oxidize fatty acids was impaired by the non-urea-adducting fatty acids.

Data on the rates at which cottonseed oil-fed controls and urea filtrate-fed rats expired $^{14}CO_2$ from ^{14}C-labeled glucose or palmitic acid are presented in table 7. Administration of the urea filtrate reduced the rate of $^{14}CO_2$ from the palmitic acid by approximately 30%. The value at every time interval except that at one-half hour was significantly different from the corresponding control value. The effect of the urea filtrate on the expiration rate of $^{14}CO_2$ from glucose was not statistically significant; however, the trend of the higher rate of glucose oxidation by the urea filtrate-fed animals was consistent with the expectation that an animal with a depressed lipid metabolism would compensate by utilizing non-lipid material at a greater rate.

The non-urea-adducting fatty acids, formed by excessive heating of fats, had profoundly deleterious effects on young rats in these studies of acute toxicity. Although the biochemical mechanism for toxic response is unknown, the data in this

TABLE 7

Percentage of glucose-^{14}C or palmitate-^{14}C converted to $^{14}CO_2$ by rats [1] fed urea filtrate

	Accumulated % of administered dose [2]						
	0.5 hr	1 hr	1.5 hr	2.5 hr	3.5 hr	4.5 hr	5.5 hr
			D-Glucose-^{14}C supplement				
CSO (7) [3]	2.77 ± 0.26 [4]	12.01 ± 0.84	21.51 ± 1.16	35.50 ± 1.60	44.17 ± 1.75	48.75 ± 1.88	52.14 ± 2.65
Filtrate (5)	3.15 ± 0.39	14.44 ± 1.42	26.18 ± 2.12	42.23 ± 2.51	50.58 ± 2.72	55.70 ± 2.79	58.98 ± 2.61
Filtrate/CSO	1.14	1.20	1.22	1.19	1.15	1.14	1.13
P value	<0.50	<0.20	<0.10	<0.05	<0.10	<0.10	<0.20
			Palmitic-1-^{14}C and supplement				
CSO (5)	2.11 ± 0.15	6.65 ± 0.25	10.96 ± 0.52	18.17 ± 0.65	23.90 ± 0.66	29.07 ± 0.56	33.48 ± 0.53
Filtrate (7)	1.60 ± 0.46	4.70 ± 0.46	7.89 ± 0.71	13.02 ± 1.14	16.85 ± 1.48	20.44 ± 1.75	23.68 ± 1.97
Filtrate/CSO	0.76	0.71	0.72	0.72	0.71	0.70	0.71
P value	<0.10	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[1] Male weanling rats fasted overnight were intubated with 1 ml/100 g body weight of urea filtrate or cottonseed oil (CSO) 1 hour before intraperitoneal injection with isotopic substrate (1 ml/100 g body wt).
[2] Palmitic-1-^{14}C acid, 10 mg (5 μCi)/ml; D-glucose-^{14}C (uniformly labeled), 80 mg (4 μCi/ml).
[3] Number of determinations.
[4] Mean ± SE.

paper characterize some of the gross changes that occurred in the animal and provide evidence that one or more of these unidentified fatty acids interfered with the oxidation of normal fatty acids.

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Utilization of a Delayed Lysine or Tryptophan Supplement for Protein Repletion of Rats[1]

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ABSTRACT Experiments were conducted to compare the utilization of graded levels of lysine or tryptophan supplement mixed in a deficient diet for protein-repletion of rats with that of the comparable supplements given apart from the deficient diet. Results demonstrate that in partially protein-depleted rats tissue protein repletion is accomplished when rats are fed for 12 hours daily an otherwise adequate but lysine-free diet and then in the following 12 hours fed a protein-free diet supplemented with lysine. Delayed tryptophan supplement, however, was not effectively utilized for tissue repletion of rats. It is suggested that the effectiveness of the delayed lysine supplement may be related to the slow turnover rate of lysine in the body or the ability of the animals to re-use some of the lysine obtained from breakdown of tissue proteins for synthesis of new proteins or both. An amino acid toxicity occurred at the high level of tryptophan supplement (1.6% DL-tryptophan), either mixed in a deficient diet or given in a protein-free diet; this was not observed with the high level of lysine supplement (5% L-lysine·HCl).

The investigations by Geiger (1) twenty years ago recognized the time factor in protein nutrition. Amino acid mixtures deficient in lysine, methionine or tryptophan did not support the growth of young rats when the missing amino acid was given apart from the deficient diet. It was concluded that effective supplementation of limiting amino acids occurred only if the time interval was less than 4 or 5 hours, when there were still sufficient quantities of the deficient diet in the intestine of the animal (2).

The studies of Yang et al. (3) showed that a lysine supplement administered 16 hours after the feeding of a lysine-deficient diet appeared to be as well-utilized for protein synthesis in young rats as a comparable supplement incorporated in the deficient diet. Further studies of Yang et al. (4) also indicated that the ineffective delayed lysine supplementation observed by Geiger (1) was due to the toxicity of the experimental diet supplemented with 2% L-tryptophan. Growth data obtained in a 4-week feeding experiment, however, showed that a delayed tryptophan supplement given apart from a tryptophan-deficient diet was not efficiently utilized by young rats.[4]

The experiments reported in the present paper were designed to compare the utilization of 3 levels of lysine or tryptophan supplement mixed in a deficient diet for tissue protein-repletion of rats with that of the comparable supplements given 12 hours later in a protein-free diet.

EXPERIMENTAL PROCEDURES

Male weanling rats of the Sprague-Dawley strain were fed a stock diet[5] ad libitum until they reached an average weight of 250 g. They were then fed an otherwise adequate but protein-free basal diet for 12 days. The basal diet (diet 1) consisted of the following: (in percent) cornstarch, 88; corn oil, 5; mineral mix (5), 4; nonnutritive cellulose,[6] 2; and vitamin-cornstarch mix (6),[7] 1.

Two experiments were conducted. Experiment 1 was designed to compare the utilization of a low, medium or high level

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[4] Yang, S. P. 1963 Utilization of delayed amino acid supplements by young rats. Proc. 6th Int. Congr. Nutr., p. 475 (abstract).

[5] Rockland Rat and Mouse Stock Diet, Tekland Inc., Monmouth, Illinois.

[6] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[7] We are indebted to the Merck and Company, Rahway, New Jersey, for generous supplies of vitamins.

of lysine supplement mixed in an otherwise adequate but lysine-deficient diet with that of the comparable supplements given 12 hours later in a protein-free diet. The minimal amount of L-lysine·HCl which Schweigert and Guthneck (7) had demonstrated to be adequate for protein repletion of the rats previously fed a protein-free diet for 12 days was chosen as the medium level of lysine supplement in the present study. The low and high levels were arbitrarily chosen to determine the effect, if any, of different quantities of lysine on the 2 methods of supplementation.

Sixty-four rats, partially protein-depleted, were divided into 8 comparable groups receiving various protein repletion diets for 7 days. They were housed individually in suspended wide-mesh, wire metabolism cages in a room maintained at constant temperature and humidity. The animals were given ad libitum water and their respective diets at 8 AM and 8 PM daily. As shown in table 1, group 1 serving as a negative control was given the protein-free basal diet (diet 1) at both 8 AM and 8 PM feedings. Group 2 receiving no lysine supplement was given diet 2 (diet 1 + 24% zein, 1.0% L-arginine·HCl, 0.4% L-histidine·HCl, 1.0% DL-isoleucine, 0.4% DL-methionine, 0.2% DL-threonine, 0.3% DL-tryptophan and 0.6% DL-valine) at 8 AM and diet 1 at 8 PM. Groups 3, 4 and 5 receiving a low, medium or high level of lysine supplement mixed in the deficient diet were given diet 3, 4 or 5 (diet 2 + 0.5, 2.5 or 5.0% L-lysine·HCl, respectively) at 8 AM and diet 1, at 8 PM. Groups 6, 7 and 8 receiving lysine supplement apart from the deficient diet, were fed diet 2 at 8 AM and diet 6, 7 or 8 (diet 1 + 0.5, 2.5 or 5.0% L-lysine·HCl, respectively) at 8 PM. All dietary additions were made at the expense of cornstarch.

TABLE 1

Experimental design of experiment 1

Group no.	Treatment	Diet fed [1]	
		8 AM–8 PM	8 PM–8 AM
1	Protein-free	1	1
2	Lysine-deficient	2	1
3	0.5% L-lysine·HCl in deficient diet	3	1
4	2.5% L-lysine·HCl in deficient diet	4	1
5	5.0% L-lysine·HCl in deficient diet	5	1
6	0.5% L-lysine·HCl delayed for 12 hr	2	6
7	2.5% L-lysine·HCl delayed for 12 hr	2	7
8	5.0% L-lysine·HCl delayed for 12 hr	2	8

[1] See text for composition of diets.

TABLE 2

Experiment design of experiment 2

Group no.	Treatment	Diet fed [1]	
		8 AM–8 PM	8 PM–8 AM
9	Protein-free	1	1
10	Tryptophan-deficient	9	1
11	0.08% DL-troptophan in deficient diet	10	1
12	0.16% DL-tryptophan in deficient diet	11	1
13	1.6% DL-tryptophan in deficient diet	12	1
14	0.08% DL-tryptophan delayed for 12 hr	9	13
15	0.16% DL-tryptophan delayed for 12 hr	9	14
16	1.6% DL-tryptophan delayed for 12 hr	9	15

[1] See text for composition of diets.

Experiment 2 was conducted to compare the utilization of graded levels of tryptophan supplement mixed in a deficient diet with that given 12 hours later in a protein-free diet. Similar to the procedures described for experiment 1, group 9 received the protein-free diet (diet 1) at both 8 AM and 8 PM feedings while group 10 received a tryptophan-deficient diet, diet 9 (diet 4 minus tryptophan), at 8 AM and diet 1 at 8 PM (table 2). Groups 11, 12 and 13 were fed the tryptophan-deficient diet supplemented with 0.08, 0.16 or 1.6% DL-tryptophan, diets 10, 11 and 12, respectively, at 8 AM and diet 1 at 8 PM. Groups 14, 15 and 16 were fed the tryptophan-deficient diet in the morning and the protein-free diet supplemented with 0.08, 0.16 or 1.6% DL-tryptophan, diets 13, 14 and 15, respectively, in the evening. The medium level of tryptophan supplement was chosen from the findings of Steffee et al. (8) on tryptophan requirement for protein repletion of the rats previously fed a low protein diet for 2 to 3 months.

During the 7-day protein repletion period, food intakes of individual rats were

recorded twice daily and body weight was recorded for each animal on the first, fourth and last days of the experimental period. The urine of each rat was collected in an 118-ml glass bottle containing 5 ml 20% HCl. At the end of the experiment, the tray and cage of each animal were rinsed with water and the rinsings were added to the bottle containing that animal's urine. Urine samples were then filtered quantitatively through a Buchner funnel and made to 250 ml with water.

Feces were removed every morning from the wire-mesh screen under the metabolism cages. They were brushed free of hair and stored at −18°. At the termination of the repletion period, the feces of each rat were transferred into a 125-ml Erlenmeyer flask containing 50 ml 20% HCl and autoclaved at 110° for 2 hours. The digest was then rubbed through a fine sieve into a 250-ml volumetric flask and made to volume with water.

At the end of the repletion period, experimental animals were anesthesized by intraperitoneal injection of 1 ml solution containing 30 mg of pentobarbital sodium.[8] The liver was immediately removed and blotted on filter paper. It was placed in a previously weighed 125-ml Erlenmeyer flask and weighed immediately. The liver was then covered with 50 ml 20% HCl, autoclaved, and made to volume by the same procedures described for the fecal samples.

The nitrogen values of the food, urine, feces and livers were determined by a macro-Kjeldahl procedure using potassium sulfate and copper sulfate as catalysts. All data were subjected to analysis of variance (9) using 1% ($P < 0.01$) as the significant level.

RESULTS

The effect of feeding a lysine-deficient diet with 0.5, 2.5 or 5.0% L-lysine·HCl supplement, either mixed in the deficient diet or given 12 hours later in a protein-free diet, on the protein repletion of rats is summarized in table 3. There was no significant difference ($P > 0.01$) in food intake between the animals receiving the protein-free diet (diet 1) and those fed the lysine-deficient diet (diet 2), in the morning (8 AM–8 PM), evening (8 PM–8 AM) or total consumption. The difference between the animals receiving either a protein-free or lysine-deficient diet and those receiving lysine supplements was significant ($P < 0.01$) for the food consumed in the morning and also the total food intake, but was not significant for the evening food intake. Method and level of lysine supplementation had no significant effect on morning, evening or total food intakes of groups 3–8. Although the nitrogen intake of groups 2–8 ranged from 1,769 to 2,766 mg/7 days, there were no significant differences among these groups.

The average weight gain of the animals receiving a protein-free or lysine-deficient diet was significantly lower than that of the animals fed a lysine-deficient diet with 0.5, 2.5 or 5.0% lysine, either mixed in the deficient diet or given 12 hours later in a protein-free diet. Similar to the findings on food intake, the weight gain of the animals was not significantly affected ($P > 0.01$) by either method or level of lysine supplementation, though there were lower weight gains at all levels with the delayed lysine supplement. There was no significant difference between the weight change of the animals fed a protein-free diet (−15 g) and that of the group receiving a lysine-deficient diet (−6 g).

There were no significant differences in liver weights among the 8 groups. The percentage liver protein content of the animals receiving a lysine-deficient diet was significantly higher than that of the group fed the protein-free diet and lower than the values of those receiving lysine supplements. When the liver protein content was expressed in milligrams per 100 g of body weight, the mean value of the protein-free group was significantly lower than the mean values of the other dietary groups. Again, method and level of lysine supplementation caused no significant difference on the liver protein content when expressed either in percentage or in milligrams per 100 g of body weight.

Nitrogen balances were significantly different between the animals fed a protein-

[8] Nembutal, Abbott Laboratories, North Chicago, Illinois.

TABLE 3

Effect of feeding protein-depleted rats for 7 days of a lysine-deficient diet with 3 levels of lysine supplement either fortified in the deficient diet or given 12 hours later in a protein-free diet

Group no.[1]	1	2	3	4	5	6	7	8
Supplementation method			In deficient diet			Delayed for 12 hours		
Level of L-lysine·HCl, %			0.5	2.5	5.0	0.5	2.5	5.0
Food intake, g								
8 AM to 8 PM	34.3 (1)[2]	39.1 (2)	48.1 (3)	51.6 (4)	51.7 (5)	43.6 (2)	47.9 (2)	46.4 (2)
8 PM to 8 AM	37.3 (1)	38.2 (1)	42.3 (1)	43.3 (1)	39.5 (1)	40.8 (6)	42.9 (7)	40.9 (8)
Total	71.6	77.3	90.4	94.9	90.8	84.4	90.8	87.3
Nitrogen intake, mg	146	1,769	2,292	2,622	2,766	2,038	2,304	2,400
Wt gain, g	− 15.0	− 6.0	7.1	10.0	9.5	− 0.8	7.6	5.5
Liver wt, g	6.27	6.72	7.06	6.85	7.00	6.73	6.90	6.85
Liver protein[3]								
%	17.03	18.70	20.12	20.14	19.75	19.42	19.99	19.99
mg/100 g body wt	519	590	641	612	613	606	621	605
Nitrogen balance, mg	− 252	259	521	792	775	510	455	496

[1] Eight rats/group.
[2] Numbers in parentheses indicate the diet numbers described in text.
[3] N × 6.25.

TABLE 4

Effect of feeding protein-depleted rats for 7 days a tryptophan-deficient diet with 3 levels of tryptophan supplement either fortified in the deficient diet or given 12 hours later in a protein-free diet

Group no.[1]	9	10	11	12	13	14	15	16
Supplementation method			In deficient diet			Delayed for 12 hours		
Level of L-lysine·HCl, %			0.08	0.16	1.60	0.08	0.16	1.60
Food intake, g								
8 AM to 8 PM	25.9 (1)[2]	10.3 (9)	51.3 (10)	48.9 (11)	30.8 (12)	14.1 (9)	18.2 (9)	30.0 (9)
8 PM to 8 AM	33.0 (1)	43.2 (1)	26.2 (1)	34.3 (1)	37.3 (1)	36.9 (13)	39.2 (14)	22.6 (15)
Total	58.9	53.5	77.5	83.2	68.1	51.0	57.4	52.6
Nitrogen intake, mg	62	520	2,449	2,418	1,580	692	914	1,460
Wt gain, g	− 15.1	− 12.4	3.5	8.5	− 1.2	− 9.0	− 11.2	− 10.6
Liver wt, g	6.05	6.38	5.89	6.45	7.07	6.21	6.18	6.19
Liver protein[3]								
%	19.62	17.96	21.42	19.95	18.52	18.42	19.16	19.66
mg/100 g body wt	630	595	599	599	637	576	606	612
Nitrogen balance, mg	− 248	− 216	589	752	406	− 40	36	133

[1] Eight rats/group.
[2] Numbers in parentheses indicate the diet numbers described in text.
[3] N × 6.25.

free or lysine-deficient diet and those receiving lysine supplements. Of particular interest is that while the animals received only 6 mg of lysine during a 7-day period from an essentially lysine-free zein diet (diet 2),[9] they were able to maintain a definite positive nitrogen balance (259 mg/7 days). Rats receiving delayed lysine supplements had significantly lower nitrogen balances than those receiving comparable supplements mixed in the deficient diet. There were also significant differences in nitrogen balances among various levels of lysine supplement. With lysine supplements mixed in the deficient diet, nitrogen balance for the low level group was lower than that of the medium or high level group; with delayed supplements, the nitrogen balances were about the same for all 3 levels.

Data obtained in experiment 2 comparing the utilization of 3 levels of tryptophan supplement mixed in a tryptophan-deficient diet with that of the comparable supplements given 12 hours later in a protein-free diet are summarized in table 4. In the morning (8 AM–8 PM), the food consumption of the animals fed a tryptophan-deficient diet (diet 9) was significantly lower than that of those receiving the protein-free diet (diet 1). Although these 2 groups were fed the same protein-free diet in the evening, the food intake of the tryptophan-deficient group was significantly higher than that of the protein-free group. This made the total food intakes of these 2 groups not statistically different ($P < 0.01$).

Tryptophan supplements administered by either method significantly increased the morning food intake of the animals over that of the groups receiving a protein-free or tryptophan-deficient diet. Administration of the tryptophan supplements in the deficient diet significantly increased both the morning and total food intake of the animals over those receiving comparable supplements 12 hours later in a protein-free diet. Animals receiving 1.6% DL-tryptophan fortified in the deficient diet had a lower total food intake than those receiving 0.08 or 0.16% DL-tryptophan supplement. The total food intakes of the 3 groups receiving graded levels of tryptophan apart from the deficient diet were similar.

There was no significant difference in weight change between the animals receiving the protein-free diet and that of the group receiving the tryptophan-deficient diet (table 4). Mixing the tryptophan-deficient diet with 0.08, 0.16 or 1.6% DL-tryptophan significantly increased the weight gain of the animals over that of the groups receiving comparable supplements 12 hours later in a protein-free diet or those receiving a protein-free or tryptophan-deficient diet. Of the rats that received a supplement in the deficient diet, the group receiving 1.6% tryptophan showed a weight loss (−1.2 g), whereas the groups receiving 0.08 and 0.16% tryptophan supplements gained 3.5 and 8.5 g, respectively.

The liver weight of various dietary groups ranged from 5.89 to 7.07 g and the liver protein content ranged from 17.96 to 21.42% or 576 to 637 mg per 100 g of body weight (table 4). Although the liver protein content when expressed in milligrams per 100 g of body weight increased with increasing levels of tryptophan supplement and the values for the animals receiving supplements in the deficient diet were greater than those for animals receiving delayed supplements, the differences were not statistically significant ($P > 0.01$).

The 7-day nitrogen balance of the animals receiving the tryptophan-deficient diet (−216 mg) was not significantly different from the value (−248 mg) of those fed the protein-free diet (table 4). The weight change and nitrogen balance of group 9 and those of group 1 observed in experiment 1 (table 3) were essentially the same, as both groups received the same protein-free diet during the repletion period. These findings demonstrated the similarity of the animals and experimental conditions of the 2 experiments conducted in the present study.

Rats receiving various tryptophan supplements showed significantly higher nitrogen balances ($P < 0.01$) than the animals fed the protein-free or tryptophan-deficient diet. Administration of tryptophan supplements in the deficient diet produced significantly higher nitrogen balances than the

[9] The lysine content was determined by a microbiological assay.

comparable supplements given 12 hours later in the protein-free diet. The mean nitrogen balance of the rats that received the high level of tryptophan supplement in the deficient diet was lower than that of the animals receiving low or medium levels of supplement in the deficient diet; but the nitrogen balance of the group receiving the high level of supplement in the protein-free diet, was higher than that of the group that received the low or medium level of supplement administered by the same method. These differences may be explained by the nitrogen intakes as there is a linear relationship between the 7-day nitrogen intakes and nitrogen balances of various dietary groups (table 4).

DISCUSSION

Results obtained in the present experiments demonstrate that rats partially protein-depleted can accomplish tissue protein repletion when fed for 12 hours daily an otherwise adequate but lysine-deficient diet and then in the following 12 hours fed a protein-free diet supplemented with lysine. Although the rats receiving lysine supplements fortified in the deficient diet retained more nitrogen during a 7-day protein-repletion period than the animals receiving comparable supplements 12 hours later in a protein-free diet, data on weight change and hepatic protein repletion did not reveal any significant difference. These findings are in agreement with our earlier observations with weanling rats (3, 4) and also those of Howe and Dooley (10) who reported that when the food intake was restricted the lysine supplementation of a gluten diet was equally effective in supporting the growth of weanling rats whether the supplement was incorporated in the gluten diet or administered by a stomach tube 6 hours after feeding. Their studies, however, showed that the administration of supplemental lysine in the gluten diet proved to be a more effective method of supplementation when the food intake was unrestricted. The food intake was not controlled in the present study.

Recently Mitchell and Morrison [10] found that the growth of young rats was retarded when the supplemental lysine was delayed for 6, 12 or 18 hours after feeding a threonine-supplemented gluten diet. It should be noted that lysine is the first-limiting amino acid of wheat gluten for growing rats, and threonine, the second-limiting amino acid. Therefore, it is reasonable to believe that the poor growth response of the animals given delayed lysine supplementation was probably due to the feeding of an amino acid-imbalanced diet (a wheat gluten diet supplemented with threonine).

The present data also demonstrate that protein-depleted rats cannot utilize the delayed tryptophan supplement as effectively as the delayed lysine supplement. The delayed tryptophan supplement supported positive nitrogen balance at the medium and high levels but did not support weight gain, whereas the delayed lysine supplements supported positive nitrogen balances at all 3 levels and supported weight gains at the medium and high levels. An otherwise adequate but tryptophan-deficient diet supported neither weight gain nor positive nitrogen balance, whereas an essentially lysine-free diet supported a definite positive nitrogen balance.

The ineffectiveness of the delayed tryptophan supplement observed in the present experiments corroborates the data of Geiger (1), Schaeffer and Geiger (11), and Yang,[11] who have demonstrated that a tryptophan supplement given apart from a deficient diet was not effectively utilized by weanling rats. Berg and Rose (12) also found that feeding half the daily allowance of tryptophan to young rats receiving a tryptophan-deficient diet at intervals of 12 hours induced better growth than when the total daily allotment was given as a single daily supplement.

The effectiveness of a delayed lysine supplement may be related to the slow turnover rate of lysine in the body or the ability of the animals to re-use some of the lysine obtained from breakdown of tissue proteins for synthesis of new proteins, or both, as suggested by Bender (13). Not only was Bender able to keep animals alive for more than 6 months with a lysine-free

[10] Mitchell, E. M., and M. A. Morrison 1965 Effect of feeding delayed supplements of lysine and threonine to wheat gluten in the weanling rat. Federation Proc., (24)2: 499 (abstract).

[11] See footnote 4.

diet, but he also obtained a net protein utilization value of 30 with weanling rats fed an otherwise adequate but lysine-free diet, while the value for a comparable tryptophan-free diet was only 17.

An amino acid toxicity occurred at the high level of tryptophan supplement (1.6% DL-tryptophan), either mixed in a deficient diet or given in a protein-free diet; this was not observed with the high level of lysine supplement (5.0% L-lysine·HCl). This difference may be due to the level of these 2 amino acids used in the experimental diets. The high level of tryptophan supplement was about 16 times the 0.09% DL-tryptophan that Steffee et al. (8) found to be adequate for effective tissue repletion of protein-depleted rats. The high level of lysine supplement, however, was only 9 times the 0.53% L-lysine·HCl established by Steffee and associates. In another study conducted at this laboratory (14), we have observed lysine toxicity when it was fed at the 12.5% level.

Positive nitrogen balances coupled with body weight losses of several dietary groups in the present study have demonstrated a typical characteristic of hypoproteinemic animals. That is, during protein-repletion, animals receiving diets containing a protein of poor quality may come into positive nitrogen balance, but continue to lose body weight (15). Nitrogen balance is the summation of many variables in the body and it is possible for an animal to be in positive nitrogen balance and yet be depleting some labile stores in tissue proteins (16).

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Microbial Species Including Ureolytic Bacteria from the Rumen of Cattle Fed Purified Diets

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ABSTRACT The rumen microbial populations were studied in cattle fed either natural or purified diets. The purified diets contained either isolated soy protein or 1 of 4 nonprotein nitrogen (NPN) sources. Four-hundred and three strains of bacteria isolated at random from a nonselective medium (264 strains) and a lactobacillus medium (139 strains) were presumptively identified and tested for urease activity. There were 28 and 2 ureolytic strains among the total bacteria isolated from the nonselective and lactobacillus medium, respectively. The ureolytic strains represented a wide variety of bacteria. Among the total population, the most predominant presumptively identified groups of bacteria grown on nonselective medium were anaerobic lactobacilli and *Bacteroides amylophilus*. The latter group's concentrations in rumen contents were higher in the steers fed either urea or urea phosphate than in steers fed biuret or uric acid. The percentage of facultative anaerobic and H_2S-producing ruminal bacteria were significantly greater for steers fed biruet than for steers fed the other NPN sources. Steers fed uric acid had significantly more cellulolytic bacteria than steers fed urea and urea phosphate and steers fed urea had significantly more amylolytic bacteria than steers fed urea phosphate. Cattle fed a 30% roughage-70% concentrate natural diet contained significantly more ruminal protozoa and gram-negative rod-shaped bacteria but contained significantly fewer cocci than cattle fed urea or isolated soy supplemented purified diets.

Much of the information which describes the ruminal microbial population has been obtained from animals fed forage or forage-concentrate diets. Little information describing the population in ruminants fed only concentrate diets is available and even less is known about the population in ruminants fed purified diets. In addition, although rumen contents possess high urease activity, attempts to isolate strictly anaerobic ureolytic bacteria from high dilutions ($> 10^{-8}$) of rumen contents have been unsuccessful. The isolation of rumen microorganisms in pure culture and the subsequent determination of the nutritional requirements and the metabolic products of the organisms should create a clearer understanding of the interrelationships between microbial species within the rumen and increase our knowledge about the symbiotic relationship between the host animal and its microbes. The following study was initiated to determine: 1) the composition of the predominant microbial population in the rumen of cattle fed purifed or 70% concentrate diets; 2) the effect of dietary nonprotein nitrogen (NPN) upon the types of microorganisms established in the rumen; and 3) the types of rumen bacteria that are ureolytic.

EXPERIMENTAL PROCEDURE

Animals and diets. In one experiment microorganisms were obtained from steers fed purified diets differing only in the nitrogen source supplied. Either urea, urea + urea phosphate, biuret or uric acid served as the nitrogen in each diet. Unstrained ruminal samples were collected 4 hours after feeding at the end of each of the periods of the 4 × 4 Latin square experiment. Animals, diets and collection procedures were the same as those used in the metabolism trial described by Oltjen et al. (1). The rumen contents were assayed, using all the techniques described in the microbial and chemical methods section.

In a second experiment, unstrained ruminal samples were taken 2 hours after feeding from cattle fed 30% roughage diets and from cattle fed isolated soy or urea-containing purified diets. Only the total (viable plus nonviable) numbers and the morphological types of protozoa and bacteria present in each sample were determined. The animals, diets and collection

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procedures were those of experiment 1 as previously described by Ørskov and Oltjen (2).

Microbial and chemical methods. The total number of culturable rumen bacteria was determined by colony counts using the anaerobic roll tube procedure of Hungate (3) as modified by Bryant and Robinson (4). A nonselective rumen fluid medium 98-5 with a 100% CO_2 gas phase (4) was one of the roll tube media used. The total number of colonies was determined from the average of counts from 4 replicate roll tubes prepared at each of 2 concentrations (0.2 and 1 ml of 10^{-8} dilution) from each rumen sample. A culturable bacterial count was also determined using the lactobacillus selective medium of Rogosa et al. (5) and the anaerobic roll tube technique (3, 4). The medium was modified to include a final concentration of 2% agar, 0.05% cysteine•HCl and a 100% CO_2 gas phase. The total number of culturable bacteria was determined from the average of the colony counts from 2 roll tubes prepared at each of 2 concentrations (1 ml of 10^{-7} and 10^{-8} dilution) after 72 hours of incubation at 38°.

Bacteria were selected at random for presumptive identification from the lactobacillus (10/sample) and the 98-5 media (20/sample) after 96 hours' incubation using the techniques of Bryant et al. (6, 7) except that a modified pH glucose medium was used (8). The pH glucose medium was prepared using rumen fluid from a steer fed a forage-concentrate diet because the growth and final pH of anaerobic lactobacilli was atypical (slow growth, high pH) when rumen fluid from steers fed the purified diets was used. A total of 403 strains (264 from nonselective medium and 139 from lactobacillus selective medium) were studied.

All bacterial strains which grew in pH glucose medium were tested for ureolytic activity. In cases where growth did not occur in the pH glucose medium, starch cellulose broth cultures from the presumptive identification tests were used as the cell source for testing ureolytic activity. The assay involved the following: After 7 days of incubation the cultures were centrifuged at 2500 × *g* for 5 minutes. The supernatant was decanted under 10% CO_2, 90% N_2 gas phase and 4 ml of a solution containing salts, maleic acid, urea, phenol red and cysteine•HCl was added. The solution was that of Jones et al. (9), modified to include 0.05% cysteine•HCl and a 10% CO_2-90% N_2 gas phase. A urease-positive strain was one that caused a deep cerise color to develop within 96 hours.

Protozoal numbers (10), total bacterial numbers and fatty acids were determined as previously described (8). Morphological types of bacteria in rumen contents were determined by microscopic examination of a gram-stained smear. Deoxyribonucleic acid (DNA) concentration in rumen contents was determined as previously described (10) except that the contents were extracted 3 times for DNA rather than once.

Tests for significance were made using *F* ratios derived from orthogonal contrasts.

RESULTS

Of the 403 strains tested, an average of 7.4% possessed urease activity. Of the 264 strains isolated from nonselective medium, 28, or 10.6% were ureolytic compared with 2, or 1.4%, for the 139 strains isolated from the lactobacillus medium. The morphology and some of the physiological characteristics of all the strains isolated together with the same characteristics for the ureolytic strains of bacteria are shown in table 1. Some strains which possessed urease activity were presumptively identified as *Propionibacterium, Bacteroides sp., Ruminococcus sp., Streptococcus bovis* and anaerobic lactobacillus, but the majority, 68%, of the ureolytic strains isolated could not be presumptively identified by the techniques used. An assay of fatty acids produced by the unidentified ureolytic strains grown in the pH glucose broth revealed no consistent volatile fatty acid(s) as end-products. The acid(s) and the number of strains producing the acid(s), respectively, from among 13 strains tested were as follows: butyric, 3; acetic, 2; butyric and acetic, 1; acetic and propionic, 2; and propionic and valeric, 1. Four strains did not produce volatile fatty acids. From among the unknown strains the 3 butyric acid-

TABLE 1

Comparison of the physiology and morphology of the ureolytic bacteria to that of the total bacteria isolated from steers fed purified diets containing different NPN sources [1]

Characteristic	% of strains isolated on:			
	Medium 98-5		Lactobacillus medium	
	All strains	Ureolytic strains	All strains	Ureolytic strains
Morphology				
Rods				
Gram-negative	40.3	35.7	17.2	0
Gram-positive or variable	39.0	21.4	70.6	100
Cocci				
Gram-negative	7.3	14.3	0.7	0
Gram-positive or variable	8.8	28.6	11.5	0
Spirochete	1.9	0.0	0.0	0
Other	2.7	0.0	0.0	0
Physiology				
Anaerobic	78.1	67.9	78.5	100
Motile	16.6	17.9	2.8	0
H_2S producers	5.7	7.1	5.8	0
Gas producers	14.7	25.0	3.2	0
Starch hydrolyzers	72.1	42.9	44.6	100
Acid producers from glucose	58.5	35.7	97.0	100

[1] The number of bacterial strains studied which were isolated from medium 98-5 from steers fed urea, biuret, urea phosphate plus urea and from uric acid were 72, 74, 54 and 64, respectively. The number of strains studied, which were isolated from lactobacillus medium, from steers fed urea, biuret, urea phosphate plus urea and from uric acid were 32, 38, 31 and 38, respectively. There were 28 and 2 strains of ureolytic bacteria isolated from the 98-5 and the lactobacillus media, respectively.

producing bacteria were the most similar as a group. They were isolated from 2 steers fed uric acid. They were cocci, 0.7 to 1.0 μ in diameter and were gram-positive, nonmotile, strictly anaerobic bacteria that were positive for gas production, negative for starch and cellulose hydrolysis, and negative for H_2S production.

The physiological characteristics of the isolated bacteria from steers fed the different nitrogen sources are shown in table 2. In making the orthogonal contrasts to determine significant differences between the flora and fauna of steers fed the different nitrogen sources the following comparisons were made. Biuret was compared with the other nitrogen sources, uric acid was compared with urea and urea phosphate, and urea was compared with urea phosphate. A greater percentage of bacteria isolated from biuret-fed steers were H_2S producers ($P < 0.01$) and more were acid producers from glucose ($P < 0.01$). When the steers were fed urea a greater proportion of the bacteria isolated utilized starch ($P < 0.05$). The cellulolytic bacterial numbers were greatest ($P < 0.05$) for steers fed uric acid and are based upon results obtained from the inoculation of diluted rumen contents (1 ml of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}) into replicate starch cellulose tubes (table 3). The proportions of ureolytic bacteria were less, although not significantly so, when the steers were fed biuret than when they were fed the other diets.

TABLE 2

Some physiological characteristics of ruminal bacteria isolated from steers fed purified diets

Characteristic	Urea	Biuret	Urea phosphate and urea	Uric acid
	% of total			
Anaerobic	75	64	87	91
Motile	19	20	15	11
H_2S producers	0	16	4	2
Gas producers	17	17	4	19
Ureolytic	6	1	15	9
Starch hydrolyzers	88	68	69	63
Acid producers from glucose	61	80	24	59

The viable bacteria, on the average, represented 10% of the total bacteria observed from the direct microscopic count (table

TABLE 3

Effect of nitrogen source on total bacteria and protozoal numbers and on bacteria with certain physiological characteristics

Rumen contents	Urea	Biuret	Urea phosphate and urea	Uric acid
Bacteria [1]				
Total/g $\times 10^{-8}$	435.0	456.0	567.0	471.0
Rods, %				
Gram-negative	32.5	38.3	18.0	37.0
Gram-positive or variable	20.5	29.0	31.3	27.3
Cocci, %				
Gram-negative	13.0	21.5	16.8	9.5
Gram-positive or variable	33.8	10.0	34.0	24.5
Spirochete, %	0.3	1.3	0.0	1.8
Viable/g $\times 10^{-8}$	48.0	64.0	30.0	53.0
Lactobacilli/g $\times 10^{-7}$	62.0	242.0	28.0	180.0
Cellulolytic/g $\times 10^{-8}$	3.8	2.8	0.9	7.8
Protozoa/g $\times 10^{-3}$	31.6	4.0	43.0	51.1
μg DNA/g	424.0	418.0	467.0	483.0
Ruminal pH	5.8	5.7	5.8	5.8

[1] The lactobacilli/g $\times 10^{-7}$ represent all the culturable bacteria which grew on the lactobacillus medium.

3). Each bacterium in the rumen contents on the average contained about 0.9×10^{-8} μg DNA. This figure does not include a correction for DNA contributed by the protozoa nor does it take into account possible DNA losses due to cell lysis. In a previous report a pure culture of *Butyrivibrio fibrosolvens* was continuously cultured. Eighty-five percent of the cells appeared to be viable and on the average, each bacterium contained 5.5×10^{-8} μg DNA (8).

The two most frequent groups of bacteria isolated from steers fed purified diets were anaerobic lactobacillus and *Bacteroides amylophilus* (table 4). These bacteria were found in 11 and 7 samples, respectively, of the 16 ruminal samples assayed and represented 18.9 and 19.3%, respectively, of the total bacteria isolated. The frequency of appearance of these bacteria varied according to the NPN fed. The flora of the biuret-fed steers contained fewer *Bacteroides amylophilus* and more facultative anaerobic bacteria (lactobacillus and *Streptococcus bovis*) than the flora of steers fed the other nitrogen sources. Except in samples from steers fed biuret, the majority of cocci could not be presumptively identified. The greatest proportion of identifiable cocci were *Streptococcus bovis* or *Peptostreptococcus elsdenii* (table 4). The latter were present in slightly greater numbers in the steers fed biuret and these produced butyric and caproic acid in pH glucose broth. These results are consistent with slightly greater quantities of caproic and butyric acid in the rumen of steers fed biuret than those fed other nitrogen sources (2).

The effect of diet on the rumen ecology of cattle fed natural or purified diets is shown in table 5. The cattle fed the natural diet (table 5) contained more protozoa than the cattle fed either the isolated soy or urea purified diet. The range in protozoal numbers was 25,300 to 328,960/g of rumen contents for the cattle fed the natural diet. Of the cattle fed purified diets only one urea-fed animal contained protozoa. The proportion of the different types of bacteria from cattle fed the natural diet was similar to that of a previous report. [1] Cattle fed natural diets contained more gram-negative rods ($P < 0.01$) and less gram-positive rods ($P < 0.05$) than cattle fed purified diets. Whether the morphological differences were due to the nitrogen source or to other nutrient differences between the purified and concentrate diets is

[1] Slyter, L. L., R. R. Oltjen and P. A. Putnam 1965 Rumen microorganisms in wheat vs. corn all-concentrate steer rations. J. Anim. Sci., 24: 1218 (abstract).

TABLE 4

Distribution of strains of groups of bacteria isolated from medium 98-5 inoculated with rumen contents from steers fed four diets

Group	% of total strains [1]				
	Urea	Biuret	Urea phosphate and urea	Uric acid	Overall
Bacteroides amylophilus	26 (2)	1 (1)	44 (2)	9 (2)	19.3 (7)
Anaerobic lactobacillus	24 (3)	20 (3)	6 (2)	23 (3)	18.9 (11)
Facultative anaerobic lactobacillus	3 (2)	20 (3)	0	0	6.4 (5)
Peptostreptococcus elsdenii	0	5 (2)	2 (1)	0	1.9 (3)
Streptococcus bovis	0	5 (2)	2 (1)	3 (2)	2.7 (5)
Bacteroides sp.	0	1 (1)	0	0	0.4 (1)
Bacteroides ruminicola	0	1 (1)	0	0	0.4 (1)
Ruminococcus albus	0	0	0	2 (1)	0.4 (1)
Ruminococcus flavefaciens	0	1 (1)	0	0	0.4 (1)
Bacteroides succinogenies	0	0	0	2 (1)	0.4 (1)
Butyrivibrio fibrosolvens	6 (1)	0	0	2 (1)	1.9 (2)
Atypical Butyrivibrio	1 (1)	0	0	0	0.4 (1)
Borrelia sp.	0	3 (2)	0	5 (1)	1.9 (3)
Unknown rods					
Gram-negative	21	15	13	22	
Gram-positive or variable	14	14	17	11	
Unknown cocci					
Gram-negative	2	1	9	8	
Gram-positive or variable	4	8	4	8	
Other	0	4	4	5	

[1] Numbers in parentheses under the different NPN sources represent the rumen samples which contained that particular bacterial group from among the total of 4 samples collected for each diet. Numbers in parentheses in the overall column indicate the sum of the appearances of each particular bacterial group for all 4 diets.

TABLE 5

Rumen ecology of cattle [1] fed a natural diet [2] or purified diets containing either urea or isolated soy protein

Group	Purified diets		Natural diet
	Urea	Soy	
Total bacteria $\times 10^{-9}$	12	12	11
Total protozoa $\times 10^{-3}$	1	0	152
Bacteria, % of total:			
Rods			
Gram-negative	42	37	64
Gram-positive or variable	22	43	10
Cocci			
Gram-negative	21	9	19
Gram-positive or variable	14	9	5
Spirochete	1	2	2

[1] Rumen samples were obtained from 10, 6 and 7 animals for the urea, isolated soy and natural diets, respectively.
[2] The natural diet contained 30% forage and 70% concentrate.

not clear. The effect might be due to the nitrogen source because cattle fed urea contained more cocci than cattle fed isolated soy, although the differences were not significant ($P < 0.10$). The isolated soy would presumably provide a nitrogen source more like that of the concentrate.

To obtain information on the ability of the media to grow bacteria, the total lactobacilli colony numbers in the nonselective medium 98-5 and the selective lactobacillus medium were compared. To do this, the percentage of pure strains isolated and presumptively identified as lactobacilli from 98-5 and lactobacillus medium were multiplied by the number of colonies per gram of whole rumen contents in each medium, respectively. The lactobacillus

numbers in the 2 media were almost identical for samples obtained from the steers fed uric acid and urea phosphate. For steers fed urea or biuret about 2 times more lactobacilli grew in the nonselective medium 98-5 than in the lactobacillus medium. These results are interpreted to indicate that the 2 media did not differ greatly in their ability to grow lactobacilli although the inclusion of rumen fluid in the lactobacillus medium might provide a more suitable medium for growing ruminal lactobacilli. The strains presumptively identified as lactobacilli which grew in lactobacillus medium inoculated with rumen contents from steers fed urea, biuret, urea phosphate and uric acid, were 94, 55, 59 and 74%, respectively.

DISCUSSION

To our knowledge this is the first time the occurrence of a wide variety of strictly anaerobic ureolytic bacteria have been isolated as pure strains from high dilutions (10^{-8}) of rumen contents. Previous attempts to isolate strictly anaerobic ureolytic bacteria from high dilutions of rumen contents have been unsuccessful (9, 1),[2,3] although several ureolytic bacterial strains were isolated from more concentrated rumen contents and several facultative anaerobic ureolytic bacteria were isolated.

In our studies bacteria with ureolytic activity were isolated in pure culture from 7 of the 16 ruminal samples. Among the bacteria of the different morphological groups isolated, relatively more cocci than rods were ureolytic. In a subsequent study,[4] however, bacteria were isolated from a mixed rumen microbial population continuously cultured and all 16 ureolytic bacteria among the 143 strains tested were rod-shaped. In another study[5] the majority of both the ureolytic and the nonureolytic bacteria isolated and presumptively identified were Ruminococci. The morphological diversity among the different strains of ureolytic bacteria isolated in these studies show that a wide variety of rumen bacteria possess ureolytic activity. Because the quantitative importance of the ureolytic bacteria isolated in the present studies relative to the total ruminal ureolytic activity was not determined and, since the techniques used may not have allowed the most active ureolytic bacteria to grow, the possibility that undetected groups of highly active ureolytic bacteria were present in the rumen population cannot be eliminated.

The percentage of urea hydrolyzing bacteria was lower for steers fed biuret than for steers fed the other NPN sources but further studies will be required to determine whether it is a consistent finding. If true, the differences might be due to differences in the metabolism of urea and biuret (12).[6]

Urea-fed steers contained a greater concentration of amylolytic bacteria, whereas the uric acid-fed steers contained a greater concentration of cellulolytic bacteria than those fed the other NPN diets. The reasons for this are not clear though in the latter case, uric acid is the least water-soluble of the 4 NPN sources fed and its rate of ruminal degradation, therefore, might be reduced and nitrogen may be available to the cellulolytic bacteria on a slow and continuous basis.

There are perhaps several reasons that biuret-fed steers should contain a somewhat different type of ruminal flora from that of steers fed other NPN diets. One is that the low NH_3 levels in the rumen of biuret-fed steers (1) prevented the growth of some of the NH_3-requiring bacteria. *Bacteroides amylophilus* which represented a small percentage of the bacteria in the steers fed biuret has been shown to require NH_3 for growth in some media (13). Ammonia has also been shown to be essential in some media for the growth of cellulolytic bacteria (13) and reduced cellulose digestion was obtained from steers fed biuret (1).

The high numbers of lactobacilli in the steers fed the purified diet were probably

[2] Muhrer, M. E., and E. J. Carroll 1964 Urea utilizing microorganisms in the rumen. J. Anim. Sci., 23: 885 (abstract).

[3] Carroll, E. J. 1960 Urea utilizing organisms from the rumen. Dissertation, Ph.D. Thesis, University of Missouri.

[4] Unpublished results, L. L. Slyter.

[5] Slyter, L. L., J. M. Weaver, R. R. Oltjen and P. A. Putnam 1967 Cellulolytic bacteria from cattle fed purified diets. J. Anim. Sci., 26: 880 (abstract).

[6] Nishihara, J., K. Shoji and M. Hori 1966 Studies on the biuret-hydrolyzing enzyme from *Mycobacterium ranae*. Biol. Abstr., 47: 4554 (abstract).

due to the high content of glucose and starch in the diet. The large number of *Bacteroides amylophilus* were probably related to the high proportion of starch. The frequency at which these particular groups of bacteria were found is probably indicative of bacterial groups which will predominate when highly fermentable purified diets are fed and does not indicate that these groups of bacteria will predominate among strains isolated from cattle fed other purified diets such as those used by Slyter et al.[7]

Most of the predominant strains of bacteria in the rumen of steers fed purified diets in this study are those that do not have a C_4 or C_5 volatile fatty acid requirement (13). Only traces of isovaleric (2, 14, 15) and isobutyric (2, 15) were found in rumen contents of sheep (14, 15) and cattle (2) fed purified diets containing urea as a nitrogen source. The apparent relationship between the growth of bacteria not requiring C_4 or C_5 fatty acids and the reduced concentrations of branched-chain fatty acid levels in the rumen may be coincidental, since C_4- or C_5-requiring bacteria have been found to be predominant among bacteria in a steer fed a urea purified diet.[8]

Although 2-methyl butyric, and isovaleric acids presumably serve as precursors for isoleucine and leucine, amino acids present in slightly greater concentration in ruminal protozoa than ruminal bacteria, the increased concentration of the branched-chain acids in the rumen fluid of cattle fed isolated soy (2) did not increase the ruminal protozoal concentration over that of cattle fed urea (table 5). The reduced ruminal pH (16, 17) in cattle fed isolated soy (2) probably prevented any stimulatory effect that the branched-chain acids or amino acids might have had on increasing protozoal numbers in the present experiment, although factor(s) other than pH may have contributed to the reduced protozoal numbers observed in cattle fed purified diets (18, 19).

Further work will be required to determine why fewer cocci grew in vitro (table 4) than were observed in direct staining of rumen contents (table 3). It may be due in part to the fact that some of the "so-called" cocci of gram-stained mixed rumen contents probably are, in fact, short rods and are so designated when observed in larger numbers such as when grown out in pure culture during the presumptive identification process.

The distribution of morphological types of rumen bacteria in cattle changed depending upon the diet fed. In this respect our results differ from those of previous reports (20, 21) where the morphology of bacteria was similar for sheep (20) fed either a semipurified or a roughage-concentrate diet and where a higher proportion of the bacteria were cocci than in the present study. A high proportion of cocci has also been shown for bulls fed a roughage-concentrate diet supplemented with urea (21).

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[7] See footnote 5.
[8] See footnote 5.

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Evaluation of Urea, Biuret, Urea Phosphate and Uric Acid as NPN Sources for Cattle

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ABSTRACT Urea, biuret, urea phosphate and uric acid were evaluated as sources of dietary nitrogen in purified diets for steers. In vivo studies with steers fed urea indicated that the ruminal microorganisms were essentially unable to degrade biuret to ammonia but degradation of uric acid to ammonia occurred to a greater extent. When the steers were fed these nonprotein nitrogen (NPN) sources for 21 days ruminal degradation to ammonia was more pronounced, especially with uric acid. Metabolism results indicated that the apparent digestibility of dry matter and gross energy were significantly greater when uric acid was compared with biuret. Acid detergent fiber digestibility was significantly less when steers were fed biuret as compared with the other NPN sources. Nitrogen retention (% of intake) was 18.4, 16.9, 12.3 and 23.1 for urea, biuret, urea phosphate and uric acid, respectively. Urinary excretion of biuret and uric acid was significantly greatest when the steers were fed these NPN sources, but while the urinary levels of uric acid were low in comparison with intake, approximately 25% of the ingested biuret was excreted in the urine. Twenty amino acids and other ninhydrin-positive compounds were significantly affected by NPN source, time after feeding or an interaction between these. Apparent blood plasma biuret was noted in significantly greater quantities, whereas blood plasma urea was present in significantly lesser quantities when steers were fed biuret. Blood plasma concentration of urea and several of the essential amino acids were significantly lower 4 hours after feeding compared with before feeding.

The ability of the ruminal microorganisms to degrade urea to ammonia and to incorporate the resulting ammonia nitrogen into microbial protein at the ruminal level is well-known. An adequate supply of readily available energy, however, is necessary before urea is the most efficiently utilized. Diets containing large amounts of roughage generally do not contain sufficient amounts of readily available energy to promote optimal utilization of urea. Therefore, the ammonia produced is frequently wasted and, in extreme cases, may be toxic to the host animal. Information is needed on other sources of nonprotein nitrogen (NPN) which can be efficiently utilized by the ruminal microorganisms and which also has the potential of being well-utilized by ruminants fed diets high in roughage. In the present experiments the purified diet technique was used to evaluate urea, biuret, urea phosphate and uric acid as NPN sources for cattle.

EXPERIMENTAL PROCEDURE

Ruminal ammonia studies. Trial 1. This trial was conducted to determine 1) the ruminal ammonia concentration resulting from the degradation of 4 NPN sources when they were included in a complete diet and placed into the rumen, and 2) whether ruminal microorganisms preadapted to urea could degrade biuret and uric acid to ammonia.

Four ruminally cannulated steers, averaging 320 kg, were fed a daily amount of feed equal to 1.3% of their body weight. The total daily feed for each animal was divided into equal amounts and offered at 8 AM and 4 PM. The steers were fed the urea diet (table 1) for 21 days before the first ruminal fluid collection. On the morning that samples were obtained, the steers were not fed and were placed in collection stalls. A ruminal ingesta sample was taken from each steer (zero time) and then each steer received one of the diets described in table 1, with the diet being placed into the rumen via the cannula. The feed was thoroughly mixed with the ruminal contents and additional samples were collected at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 hours after the zero sampling. Ammonia (1) and pH determinations were made on

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TABLE 1

Composition and analysis of experimental diets

	Urea	Biuret	Urea + urea phosphate	Uric acid
	Diet A	Diet B	Diet C	Diet D
	%	%	%	%
Cornstarch	28.4	28.3	27.0	27.7
Glucose monohydrate [1]	28.4	28.3	27.0	27.7
Wood pulp [2]	30.0	30.0	30.0	30.0
Urea [3]	4.7	0.0	2.5	0.0
Biuret [4]	0.0	4.9	0.0	0.0
Urea phosphate [5]	0.0	0.0	5.0	0.0
Uric acid	0.0	0.0	0.0	6.1
Mineral mix [6]	6.4	6.4	6.4 [7]	6.4
Refined soybean oil	2.0	2.0	2.0	2.0
Choline chloride	0.1	0.1	0.1	0.1
Vitamins [8]	+	+	+	+
Chemical analysis, % dry matter				
Crude protein	14.3	14.0	14.4	14.1
Acid detergent fiber	27.9	27.3	28.7	28.6
Ash	6.2	5.9	6.6	6.3
Phosphorus	0.7	0.7	1.0	0.7

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Grade CBR-20. International Filler Corporation, North Tonawanda, New York (analyzed 82% acid detergent fiber; 17% lignin; 1% ash).
[3] Feed grade.
[4] Biuret, 86.0%; triuret, 10.2%; urea, 2.7%; cyanuric acid, 1.1%.
[5] Analyzed 17.7% nitrogen and 19.6% phosphorus.
[6] Mineral mixture contained: (in percent) $CaHPO_4$, 49.987; K_2CO_3, 28.737; $MgSO_4$, 11.010; NaCl, 8.478; $FeSO_4$, 0.750; $MnSO_4 \cdot H_2O$, 0.112; $Na_2B_4O_7 \cdot 10H_2O$, 0.361; $ZnSO_4 \cdot 7H_2O$, 0.529; $CuCO_3$, 0.030; KI, 0.003; $CoCl_2 \cdot 6H_2O$, 0.001; MoO_3, 0.001; and Na_2SeO_3, 0.001.
[7] $CaCO_3$ replaced $CaHPO_4$ in the mineral mixture.
[8] Contained 8,800 USP units vitamin A as vitamin A palmitate; 1,100 USP units vitamin D as D-activated plant sterol and 21 IU vitamin E as *d*-α-tocopheryl acetate/kg diet.

each strained sample. The procedure of feeding via the cannula and sample collection was repeated 3 times at weekly intervals and the steers were fed the urea diet (table 1) between each collection. Each collection represented one period of a 4 × 4 Latin square and the experimental diets were administered accordingly.

The steers were not allowed to drink water during the collection but 500 ml of water were placed into the rumen after each hourly collection in an effort to maintain water balance. In this trial all of the nitrogen in diet C (table 1) was supplied as urea phosphate and the amount of each diet placed into the rumen was the same as the previously fed urea diet. The nitrogen sources were tested on an isonitrogenous basis. By placing the complete diet into the rumen of nonfasted animals, a more realistic approach as to natural conditions was obtainable.

Trial 2. Trial 2 was conducted to determine whether a 21-day adaptation (defined as the changes in the microbial population which enable it to more efficiently utilize the available substrate) by the steers to the biuret and uric acid diets (table 1) would affect the ruminal degradation of these NPN sources as measured by ammonia concentration. The steers, feeding and sampling procedures used in trial 1 were used again in this single reversal experiment. Ruminal samples were collected at the end of a 21-day adaptation period and again 3 days later for each period. During the last period one steer went off feed while being fed the biuret diet and, therefore, neither the uric acid nor the biuret data from this steer were included in the experimental results.

Metabolism and plasma amino acid study. A metabolism trial, designed as a 4 × 4 Latin square, was conducted using a 32-day adjustment period followed by a 7-day collection period for each of the 4 periods. Hereford steer calves, averaging 165 kg were fed a daily amount of feed equal to 1.3% of their body weight and each daily allotment of feed (table 1) was divided into 2 equal portions and fed at 8 AM and 4 PM.

Nitrogen (2) and phosphorus (3) determinations were made on the feed, feces and urine. Dry matter, acid detergent fiber (4) and gross energy determinations were made on the feed and feces. Uric acid (5, 6) and biuret were determined on the urine. For the biuret analysis the urine samples were filtered through Millipore filter paper and determined to be protein-free by adding 1 ml of urine to 5 ml 1% picric acid and checked for the absence of a precipitate. Then 2 ml of the filtered urine were placed in a 25-ml test tube, 8 ml of 0.85% NaCl added, followed by 10 ml of Weichselbaum's reagent (7). Then 0.5 ml of 50% NaOH was added followed by shaking. The colorimetric readings were made 45 minutes later against a reagent blank at 550 $m\mu$ and using a 2.5-cm diameter cuvette.

Jugular blood samples (heparinized) were obtained for plasma amino acid analysis (8) from each steer before the morning feeding (16 hours after evening feeding) and again 4 hours after the morning diet was consumed. The blood samples were taken after each metabolism collection period and after the steers had consumed the test diets for 40 days. Norleucine was used as an internal standard to determine the amino acid losses during sample preparation and the amino acid determinations were adjusted accordingly.

Ruminal samples were obtained from each steer by stomach tube at the end of each collection period. Each sample was taken 4 hours after the morning diet was consumed and consisted of at least 1 liter of fluid. Ammonia (1) and pH determinations were made on the strained ruminal fluid.

RESULTS AND DISCUSSION

Ruminal ammonia studies. Trial 1. The results of this trial (figs. 1 and 2) indicate marked differences in the degradation of

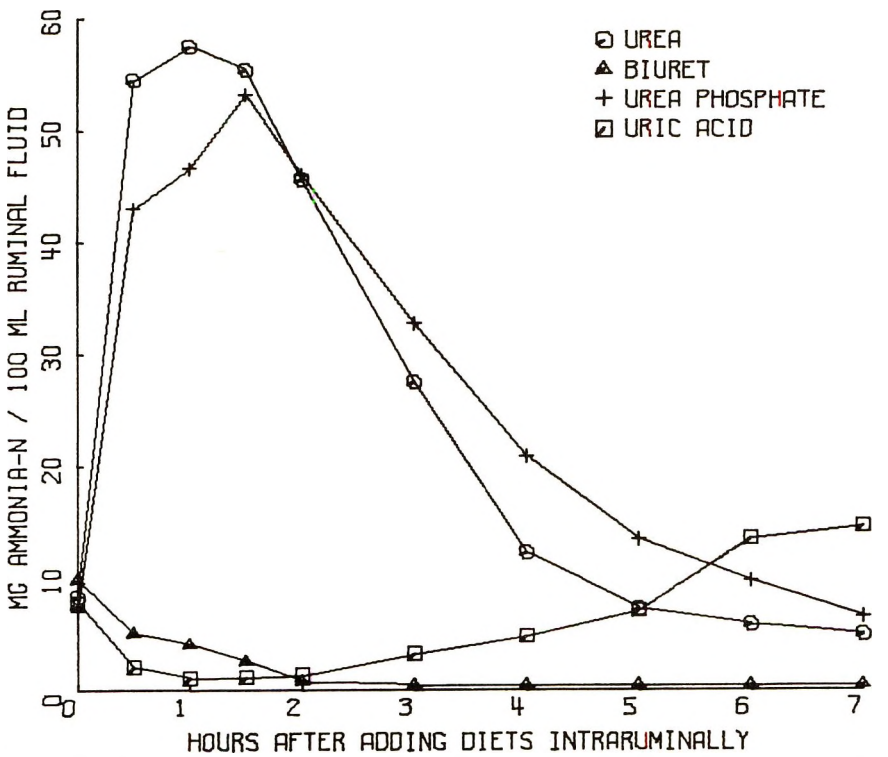

Fig. 1 Ruminal ammonia curves which are the result of placing diets containing different NPN sources into the rumens of steers preadapted to urea. Each line represents the average of 4 animal observations.

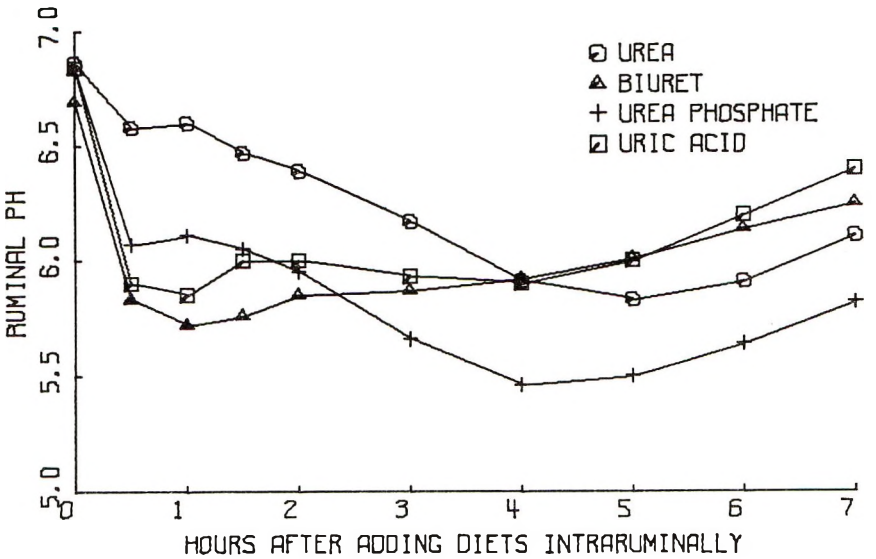

Fig. 2 Ruminal pH curves which are the result of placing diets containing different NPN sources into the rumens of steers preadapted to urea. Each line represents the average of 4 animal observations.

the different NPN sources. Urea and urea phosphate degradation resulted in similar ruminal ammonia patterns as indicated by the rapid rise in ammonia levels after feeding and a lowering to presampling levels within 6 hours after placing both the diets into the rumen. However, the peak ammonia concentration for urea phosphate was slightly lower than for urea and occurred about 30 minutes later. The pH patterns (fig. 2) were similar but averaged about 0.5 unit lower with urea phosphate. This indicates the acidic nature of urea phosphate. It has been shown that there is a positive relationship between increasing ruminal pH and the rate of passage of ammonia through the ruminal wall (9) and ammonia toxicity (10) and, therefore, the lower ruminal pH exhibited with urea phosphate may indicate a potentially less toxic compound. Urea phosphate (11) and diammonium phosphate (12) have both been found to be less toxic to sheep than urea on an equal nitrogen basis.

Biuret and uric acid were slowly degraded to ammonia in the rumen (fig. 1). However, after a 2-hour latent period, ammonia concentration increased with uric acid. While both urea and urea phosphate are very soluble in ruminal fluid, biuret and uric acid are not nearly as soluble and this may be the reason for a delayed degradation in the rumen. That ruminal fermentation was proceeding at a rapid rate with all diets is shown by the ruminal pH values (fig. 2). Quite possibly ammonia uptake was also rapid with all diets and, therefore, the interpretation that the concentration of ammonia represents the amount of NPN degraded at any given time may be misleading.

Ruminal pH was lowest 1 hour after placing the biuret and uric acid diets into the rumen, whereas it was lowest 4 to 5 hours later with the other diets. The pH values are probably higher, at least in part, due to the higher ruminal ammonia levels.

The data from this trial indicate that ruminal microorganisms preadapted to urea are essentially unable to degrade biuret to ammonia to any significant degree but ruminal adaptation and degradation of uric acid appear to be taking place after a 2- to 3-hour period in the rumen.

Trial 2. The data obtained from the trial indicate that the ruminal microorganisms were able to degrade uric acid to amomnia more quickly (fig. 1 vs. 3) after the 3-week adaptation. The ammonia release with uric acid, however, was slower

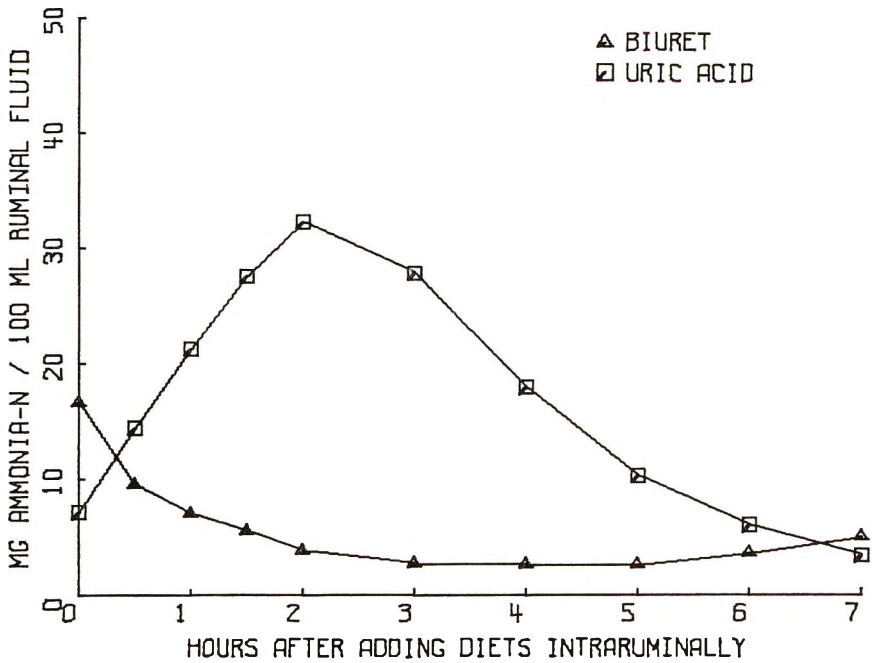

Fig. 3 Ruminal ammonia curves which are the result of placing diets containing biuret or uric acid into the rumens of steers preadapted to biuret or uric acid. Each line represents the average of 6 animal observations.

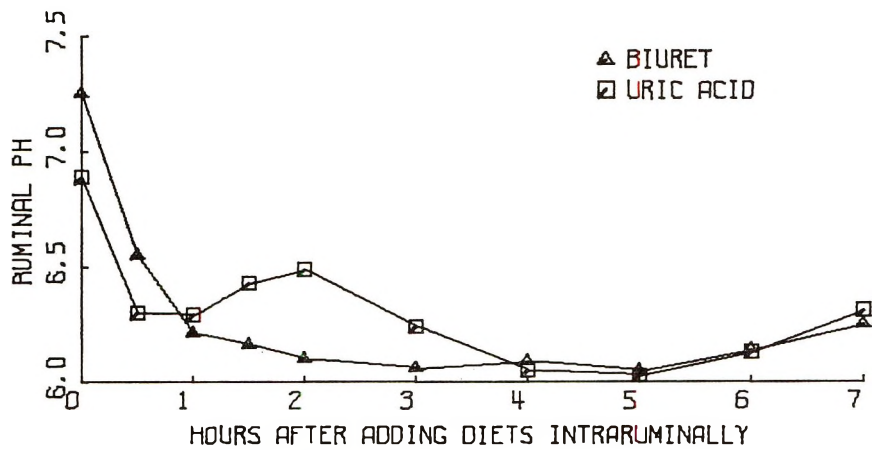

Fig. 4 Ruminal pH curves which are the result of placing diets containing biuret or uric acid into the rumens of steers preadapted to biuret or uric acid. Each line represents the average of 6 animal observations.

than with urea or urea phosphate (fig. 1) and suggests a more favorable pattern for efficient NPN utilization. The ruminal pH pattern for uric acid (fig. 4) differed from that in figure 2. The marked rise in pH in this trial at 2 hours was presumably due to the greater ruminal ammonia concentration at this time.

With respect to biuret, the presampling ammonia (zero time) values were 6.8 mg higher than in trial 1 and although a low level of 3 mg was reached, this was also

higher than in trial 1. To obtain diurnal information and to determine whether the ruminal ammonia concentrations would return to the presampling levels, one set of steers (monozygotic) were sampled for 15 hours. Ruminal ammonia nitrogen (mg/100 ml fluid) at 9, 11, 13 and 15 hours for the biuret and uric acid fed steers, respectively, were 5, 2; 8, 3; 12, 4; 13, 6. Ruminal pH appeared to follow a rising pattern similar to that for ruminal ammonia.

The interpretation of the data obtained from trials 1 and 2 indicates that bacterial uricase may be induced within hours, whereas bacterial biuretase is either more slowly induced or is increased by a shifting in the microbial population after biuret feeding. The data also suggest that even after extended feeding of biuret, little ammonia is produced, but this interpretation is complicated by the possibility that a slow, steady rate of ammonia release may result from degradation of biuret with little ammonia accumulation. It is also possible that the microorganisms may use biuret without the release of ammonia into the ruminal fluid. Johnson and McClure (13) reported that microorganisms taken from sheep "adapted" to urea or biuret failed to release ammonia from biuret when incubated in vitro. However, Campbell et al. (14) reported that there appeared to be a definite increase in the quantity of ammonia appearing in the rumen from biuret after adaptation. Ewan et al. (15) reported that nitrogen balance was significantly improved during a metabolism trial in lambs fed biuret but not in lambs fed urea if they were dosed with ruminal ingesta from other sheep fed the same NPN source. These results indicate the necessity for the development of a ruminal flora capable of utilizing biuret.

Metabolism and plasma amino acid study. The apparent digestibility of dry matter and gross energy were both greater ($P < 0.05$) when uric acid was compared with biuret in the purified diets. Ewan et al. (15) reported that the apparent digestion coefficient for dry matter was depressed when biuret was compared with urea in lambs.

The apparent digestibility of the acid detergent fiber was depressed when the steers were fed biuret compared with urea ($P < 0.05$), urea phosphate ($P < 0.05$) and uric acid ($P < 0.01$). Anderson et al. (16) reported that biuret was inferior to urea in promoting cellulose digestion. The lambs in their study, however, were adapted to biuret only 10 days whereas even after a 32-day adaptation in the present study the digestibility of acid detergent fiber was markedly depressed with biuret. Slyter et al. (17) studied the ruminal microorganisms from the present steers and reported that the steers fed uric acid had the greatest concentrations of cellulolytic bacteria, whereas biuret and urea phosphate promoted the lowest concentrations of the cellulolytic bacteria. Johnson and McClure (13) and McLaren et al. (18) have reported marked depressions in cellulose digestibility when biuret-fed wethers were compared with urea-fed wethers. However, in other studies where a 3-week or longer feeding period has been allowed, little difference in cellulose digestion has been found between urea and biuret fed ruminants (14, 19, 20). Data from the ruminal study (fig. 3) indicate that biuret should have a favorable ammonia pattern for high roughage diets, but if the digestibility of the fibrous portion of the diet is depressed, its value as an NPN source for roughage diets will be lessened. It is possible that the ruminal ammonia concentration in the steers fed biuret may have been too low to promote optimal cellulose utilization (17).

The apparent digestibility of the different NPN sources was not significantly different. Other workers (16, 18–20)[1] have reported that the apparent digestibility of urea was significantly greater than that for biuret while similar digestibilities have also been reported (13, 14). It has been shown that the digestibility of biuret increases with time (13, 18), presumably until adaptation by the animal is essentially complete. The urinary nitrogen losses were greatest (nonsignificant) for biuret

[1] Welch, J. A., G. A. McLaren, G. S. Smith, C. D. Campbell, D. C. Shelton and G. C. Anderson 1956 The relative value of biuret, creatine, soybean protein and other nitrogenous materials for lambs in digestion and nitrogen metabolism trials. J. Anim. Sci., 15: 1265 (abstract).

TABLE 2

Metabolic and ruminal data of steers fed the experimental diets

Criteria [1]	Urea	Biruet	Urea + urea phosphate	Uric acid	SE [2]
Digestibility, % intake					
Dry matter	70.1	67.9	71.1	74.4	1.5
Gross energy	68.8	62.4	69.6	70.0	1.6
Acid detergent fiber	45.1	26.0	41.6	48.8	3.7
Nitrogen, % intake					
Fecal	35.4	29.9	30.3	31.7	2.4
Urinary	46.2	53.2	57.4	45.2	3.4
Retention	18.4	16.9	12.3	23.1	5.0
Urinary excretion					
Biuret, g/day	2.8	28.8	3.6	3.1	2.9
Uric acid, g/day	0.2	0.3	0.2	0.6	0.1
Phosphorus retention					
% of intake	23.7	27.0	18.1	27.2	3.6
g/day	3.5	4.2	3.8	4.1	0.6
Ruminal data					
Ammonia-N/100 ml, mg	14.9	3.5	21.4	17.9	2.0
pH	5.7	5.8	6.0	5.8	0.2

[1] Each value is the average of 4 steers.
[2] SE of means.

and urea phosphate and although the digestibility of these NPN sources was the highest (table 2), the utilization of the degraded nitrogen was not as good as with the other NPN sources.

Nitrogen retention was almost twice as great with uric acid as with urea phosphate but the difference was not statistically different due to an abnormally high retention (32%) by one steer when fed urea phosphate. The reason for the poor nitrogen retention with urea phosphate is not clear but possibly the excess phosphorus intake adversely affected the utilization of the degraded nitrogen as evidenced by the higher urinary nitrogen values. Perez et al. (11) reported little difference in nitrogen retention between urea and urea phosphate when these made up 40 to 45% of the total nitrogen in a natural diet. There was a slight depression in nitrogen retention when biuret was compared with urea and this is in agreement with other research (13, 14, 19). However, biuret has also been found to be equal (14, 18) or definitely inferior (13, 16, 20) to urea as a nitrogen source for ruminants. Approximately 10% of the nitrogen in the present biuret source was triuret while 1% was cyanuric acid. South African research (21) indicates little difference in the nitrogen retention of sheep fed diets supplemented with biuret, triuret or cyanuric acid.

The daily excretion of apparent biuret was much greater ($P < 0.01$) when steers were fed the biuret diet compared with their being fed any of the other diets. The steers consumed over 95 g of biuret each day and excreted approximately 29 g daily in the urine. This urinary excretion level is approximately twice that reported by Hatfield et al.[2] in which steers consumed 90 g of biuret and excreted 13 g of biuret daily. A high urinary excretion of biuret has also been reported with sheep (19, 22). The daily excretion of uric acid was greater ($P < 0.05$) when the steers received the uric acid diet as compared with when they received the urea or urea phosphate diets. That uric acid was readily degraded is apparent because approximately 140 g were consumed daily by the steers fed this diet whereas only 0.6 g was excreted daily in the urine.

Phosphorus retention, expressed on a percentage of intake basis, was less (not significantly) with the urea phosphate diet than the other diets. However, due to the increased phosphorus intake with this diet the grams of phosphorus retained was similar with all diets. Perez et al. (11) reported that phosphorus from urea phosphate was as well-utilized as that from dicalcium

[2] Hatfield, E. E., R. M. Forbes, A. L. Neumann and U. S. Garrigus 1955 A nitrogen balance study with steers using urea, biuret and soybean oil meal as sources of nitrogen. J. Anim. Sci., *14*: 1206 (abstract).

phosphate. The present results may indicate a somewhat depressed utilization but the results are confounded with level of phosphorus intake. Ruminants utilize the phosphorus in diammonium phosphate as well as phosphorus from other sources (12).

Ruminal ammonia concentration 4 hours after feeding was lower when steers consumed the biuret diet compared with the urea phosphate ($P < 0.01$), uric acid ($P < 0.01$) and urea ($P < 0.05$) diets. These results agree closely with those obtained in the ruminal studies (figs. 1 and 3) for the adapted animals at this particular time after feeding and indicate the validity of placing the complete diet into the rumen. The ruminal pH values were similar and not significantly different with treatment. Data on the ruminal volatile fatty acids have been reported (23). In general the volatile fatty acid patterns were similar but with all NPN sources there were depressed concentrations of the branched-chain volatile fatty acids.

The concentrations of the free amino acids and other ninhydrin-positive compounds in the blood plasma of the steers is shown in table 3. In general, the values reported in this study are higher than those previously reported for similarly fed steers (8). However, the present values were adjusted using an internal standard and should be more representative of the true concentrations.

Blood plasma urea was lower ($P < 0.05$) when steers were fed the biuret diet compared with the other diets. This agrees with a previous study (13) in which it was reported that the amount of urea in the blood of sheep fed biuret was less than

TABLE 3

Free amino acids and other ninhydrin-positive compounds in the blood plasma of steers fed the experimental diets before feeding and 4 hours after feeding

Criteria [1]	Urea		Biuret		Urea + urea phosphate		Uric acid	
	0	4 hours	0	4 hours	0	4 hours	0	4 hours
				μmole/100 ml				
Phosphoserine	0.3	0.4	0.5	0.4	0.3	0.4	0.4	0.3
Glycerophosphoethanolamine	0.2	0.3	0.1	0.3	0.3	0.4	0.6	0.4
Phosphoethanolamine	0.2	0.4	0.3	0.3	0.3	0.3	0.5	0.4
Taurine	1.3	1.2	1.4	1.2	1.2	1.1	1.1	1.1
Urea	456.7	580.9	260.6	236.7	460.8	627.0	446.3	456.7
Biuret	100.0	99.7	315.8	323.2	92.4	62.1	100.7	106.0
Hydroxyproline	2.6	2.1	2.7	2.6	2.1	1.8	3.4	2.7
Aspartic acid	0.6	0.6	0.6	0.7	0.5	0.7	0.7	0.7
Threonine	3.8	3.1	2.8	2.3	3.0	2.7	3.8	3.5
Serine	18.9	15.4	28.5	25.3	17.1	14.5	14.3	13.6
Asparagine and glutamine	24.2	35.3	21.0	19.2	22.7	31.6	21.9	31.2
Proline	5.8	4.6	6.0	5.2	5.6	4.3	6.6	5.5
Glutamic acid	9.2	10.1	9.9	11.5	7.1	8.6	8.0	9.9
Citrulline	7.0	8.2	7.5	5.2	6.9	7.5	6.9	6.9
Glycine	53.3	39.0	68.5	58.4	53.4	34.0	51.9	48.2
Alanine	15.1	14.1	16.7	14.3	14.3	13.0	19.2	19.1
α-Amino-*n*-butyric acid	0.8	0.6	0.8	0.5	0.8	0.5	1.0	0.9
Valine	13.4	11.0	12.7	11.2	12.2	9.7	16.0	13.9
Cystine	1.2	1.1	1.4	1.2	1.4	0.9	1.4	1.5
Methionine	1.3	1.2	1.2	0.8	1.2	1.0	1.5	1.4
Isoleucine	7.4	5.4	6.1	4.8	7.3	5.2	8.7	7.2
Leucine	6.3	4.0	6.6	4.8	6.2	3.7	7.9	6.1
Tyrosine	2.5	1.9	1.9	1.3	1.6	1.9	2.3	2.4
Phenylalanine	2.6	2.1	2.1	1.8	2.6	2.0	3.0	2.8
Ornithine	4.9	4.0	4.4	3.8	5.0	4.3	4.2	3.6
Ammonia	20.9	23.6	22.1	20.3	20.7	25.1	26.1	20.7
Lysine	7.4	5.1	6.7	4.4	7.6	5.6	7.6	6.3
Histidine	7.1	7.2	7.5	6.7	7.4	6.8	7.2	7.2
Carnosine	1.0	1.0	0.9	1.0	0.8	0.9	1.1	0.9
Arginine	7.2	7.3	6.7	4.8	6.5	7.1	6.6	7.0

[1] Each value is an average of 4 steers.

half of that found in sheep fed urea. In the present study, the urea concentration was greater ($P < 0.01$) 4 hours after feeding than before feeding with all diets except where an interaction ($P < 0.05$) was apparent due to the decrease in urea concentration with the biuret diet 4 hours after feeding.

A ninhydrin-positive compound was detected and designated as biuret on the basis that it eluted from an ion-exchange column at the same position as a biuret standard. Biuret was detected in greater concentrations ($P < 0.01$) in the plasma when the steers consumed the biuret diet compared with the other diets. Biuret concentration was only slightly influenced by time. The plasma concentrations of biuret were unexpectedly high with the other NPN diets; and in reviewing the amino acid chromatograms from a previous study (8) biuret was detected at 50 μmoles/100 ml plasma when steers were fed a purified diet containing isolated soy protein. We have also detected biuret in the blood plasma of cattle fed various natural diets and thus it appears that it may be a normal endogenous constituent of the plasma.

Blood plasma ammonia was not significantly affected by diet or time but an interaction ($P < 0.05$) was found. The similar values with respect to diet were unexpected in view of the ruminal ammonia patterns in trials 1 and 2. The diet $\times$ time interaction for plasma ammonia was probably due to the more pronounced increase in ruminal ammonia with the urea diets. The increased blood urea values 4 hours after feeding the urea diets probably indicate the conversion of ammonia to urea in the liver. A diet ($P < 0.01$) and a diet $\times$ time interaction was detected with citrulline, whereas a time ($P < 0.01$) difference was observed with ornithine. Both of these compounds are involved in the "ornithine cycle" in which ammonia is detoxified and converted to urea (24). The ornithine concentration decreased after feeding all diets, while citrulline decreased only with the biuret diet at this time. Asparagine plus glutamine concentration increased ($P < 0.01$) with time with all except the biuret diet.

Amino acids showing significant differences ($P < 0.01$ or $P < 0.05$) due to diet were: hydroxyproline, serine, alanine, methionine, isoleucine, tyrosine and phenylalanine. Of the NPN sources studied, uric acid promoted the greatest nitrogen retention and when the steers were fed this diet serine was detected in lowest concentrations, whereas all the other previously mentioned amino acids significantly different with diet ($P < 0.01$ or $P < 0.05$) were found in greatest concentrations in the plasma. Furthermore, it appears that in general, nitrogen retention with the other NPN sources was directly related to the plasma concentration of these significantly different amino acids. Serine (8) and glycine [3] (25) have been detected in greater quantities in the blood plasma of cattle fed urea-containing purified diets as compared with natural diets.

Blood plasma concentrations of threonine, serine, proline, glycine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine and lysine were all found to be significantly lower ($P < 0.01$ or $P < 0.05$) 4 hours after feeding than before feeding. The concentration of glutamic acid, however, significantly increased with time after feeding. A diet $\times$ time interaction was found with tyrosine in which the concentration increased with time with the urea phosphate and uric acid diets while it decreased with the other 2 diets.

Leibholtz (26) reported that the blood plasma amino acid concentrations of sheep fed natural diets increased two- to tenfold shortly after feeding as compared with prefeeding levels. However, the infusion of a starch-glucose mixture into the rumen of sheep at the usual feeding time resulted in reduced plasma amino acid concentrations (27). This depression was most apparent with defaunated sheep. The nature of the present study in many respects resembles this research because the present purified diet contains a high percentage of readily available energy and rumen protozoal numbers in steers fed this diet have been significantly less than with steers fed a natural diet (17, 28). Theurer et al. (29)

[3] Oltjen, R. R., and J. Bond 1967 Reproduction by cows raised on a protein-free diet. J. Anim. Sci., 26: 225 (abstract).

studied the diurnal amino acid pattern in the portal and jugular plasma of sheep fed a semipurified diet containing soybean meal and reported that the amino acid concentrations tended to be lowest 4 to 6 hours after feeding.

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Heat-Labile Growth-Inhibiting Factors in Beans (*Phaseolus vulgaris*)

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ABSTRACT A comparative study in vitro was made of enzyme-inhibiting and hemagglutinin activities, and the effect on rat growth of 5 varieties of kidney beans (*Phaseolus vulgaris*). Extracts of 2 bean samples were active in agglutinating rabbit blood cells and toxic when fed to growing rats. Diets prepared with these seeds supplemented with methionine caused weight loss and death when fed to rats alone or with a supplement of 10% enzymatically or acid-digested casein. Hemagglutinating activity was observed in the feces of rats fed the raw bean diets. The possibility that the hemagglutinins are at least partly responsible for the toxic effects was examined. Three other samples of kidney beans had no significant hemagglutinating or lethal effect. Rats fed the raw seed meals supplemented with methionine did not gain weight but grew well with a similar diet supplemented with enzymatically digested casein. Supplements of 10% casein, 1% Na glutamate, or 10% acid-digested casein did not improve growth significantly but the latter did when tryptophan was added. Antitrypsin and antiamylase activities were low or absent in some of the seeds and high in others, and did not appear to be directly related to the growth inhibition observed. The low growth-promoting action of the hemagglutinin-free beans might be explained by low digestibility and an enzyme-inhibiting activity of the bulk proteins different from that of the trypsin or amylase inhibitors.

The existence of a marked inhibitory action on the growth of experimental animals fed diets containing various raw legumes, especially beans or soybeans, has been well-established. Biochemically active factors defined by their in vitro action have been observed and related to the anti-nutritional effect, notably trypsin inhibitors and hemagglutinins or lectins. The extensive literature has been reviewed by Liener (1).

The relative importance of these 2 factors and the existence of other ill-defined growth inhibitors is still subject to controversy. Although a number of papers have been published in recent years on this problem, progress in this field has been slow. Several factors may be responsible for this situation: The isolation of purified fractions with only one biochemical activity in amounts large enough for toxicological studies is difficult; a purified fraction incorporated into a non-toxic diet may have a different effect from that of a combination of active principles occurring naturally in the seeds; the raw legumes or fractions used were not always sufficiently defined with respect to the different biochemical activities present; and different legume species and even varieties of one species may vary considerably in respect to different biochemical and biological activities, a fact often neglected in this type of investigation.

In the present experiments we investigated the different varieties of one species of legumes that are free from or low in one or the other of the biochemically active factors suspected of being related to toxicity. The growth-promoting or toxic effects of diets prepared with equal amounts of the different ground seeds with known hemagglutinin and enzyme inhibitor content were compared in an attempt to relate these factors and the nutritional properties without the need of fractionation.

MATERIALS AND METHODS

The legume seeds were purchased at a local market and finely ground in a hammer mill. One part of them was soaked in water for 2 to 3 hours, autoclaved for 30 minutes at 118°, fan-dried, and then reground. The experimental diets contained, unless stated otherwise, the following ingredients: (in percent) ground legume seed meal, 40; corn oil with 0.2% percomorphum oil, 5; salt mixture USP XVI, 4; DL-methionine, 0.3; and the following vitamins (2) per

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TABLE 1

Biochemical activities in extracts of different beans

	Rabbit blood	Rat blood	Trypsin inhibitor	Amylase inhibitor
	hemagglutination units [1]		*units*	*units*
Red bean (*Phaseolus vulgaris*)	500	20	3.8	0.77
Black bean (*P. vulgaris*)	400	8	32.1	0.65
White bean (*P. vulgaris*)	3	0	3.3	2.44
Tapiramo bean (*P. vulgaris*)	0	0	20.2	1.86
Mottled bean (*P. vulgaris*)	0	0	46.0	0
Soybean (*Glycine maxima*)	80	0	28.3	

[1] All activities are expressed as units/milligram of extracted protein (N × 6.45); hemagglutination activity is expressed as the number of milliliters of a 0.2% erythrocyte suspension agglutinated by 1 mg protein (N × 6.45) of the corresponding extract.

100 g: (in milligrams) thiamine·HCl, 0.3; riboflavin, 0.3; pyridoxine·HCl, 0.2; Ca pantothenate, 0.2; niacin, 2; folic acid, 0.025; biotin, 0.010; inositol, 10; choline·HCl, 100; and cassava starch to make 100. A commercial enzymatic casein digest,[2] an acid casein digest,[3] or other supplements were added at the expense of starch. Three of the diets contained no seed meal. The kidney bean diets without added casein contained 8 to 9% protein (N × 6.25).

The rats were descendants of the Sprague-Dawley strain bred in this laboratory for over 20 years. In the first experiment, of 2 weeks' duration, male animals weighing 45 to 55 g were used. In experiments 2 and 3, three males and 3 females, weighing 35 to 45 g, were fed the respective diets for 28 days, with the exception of those dying before the end of the experiment. Young from different litters were distributed at random among the different groups. All animals were kept in individual screen-bottom cages and received food and water ad libitum. The surviving animals were killed at the end of each experiment and the pancreas and spleen removed and weighed immediately.

For the in vitro tests, extracts were prepared by allowing 10 g of finely ground seeds to stand in 100 ml of 0.85% NaCl solution for 2 hours with occasional stirring, and then filtering. Nitrogen content of these extracts was determined by the micro-Kjeldahl method.

Hemagglutination activity was studied in the ground seeds and in the feces of some of the experimental animals with washed rabbit or rat blood cells by the serial dilution technique used in earlier experiments (2) and expressed as the number of milliliters of a 0.2% erythrocyte suspension agglutinated by 1 mg of protein (N × 6.25) of the corresponding extract. Trypsin inhibitor activity was measured by the method of Kunitz (3), using casein as substrate. The method described by Bernfeld for the measurement of amylase activity (4) was used for the determination of amylase inhibitors. One unit was defined as the number of milligrams of protein in a seed extract that reduced to 50% the activity of the quantity of pancreatic amylase [4] capable of releasing 15 mg maltose from 1 ml of 1% starch solution in 3 minutes at 25° after 8 minutes of pre-incubation of amylase and inhibitor.

Nitrogen absorption was calculated in each animal of experiments 2 and 3 by measuring food consumption and fecal nitrogen excretion. Nitrogen was determined by macro-Kjeldahl method.

RESULTS

The results of several of the in vitro tests on biochemical activities of the bean varieties used are presented in table 1. Extracts of red and black kidney beans agglutinated both rat and rabbit blood cells, whereas soybean extracts were active only with rabbit blood; the other extracts had little or no hemagglutinating activity under the present experimental conditions.

Mottled and black kidney beans and soybeans had considerable trypsin inhibitor

[2] Bacto-casitone, Difco Laboratories, Detroit.
[3] Bacto-casamino-acids, Difco Laboratories.
[4] Pangestin, E. Merck, Darmstadt.

TABLE 2

Performance of rats fed different experimental diets [1]

No.	Diet	Wt change/day	Pancreas wt/ body wt × 100	Spleen wt/ body wt × 100
		g		
1	Raw black beans	− 1.8 ± 0.58 [2,3,4]	0.47 ± 0.13 [4]	0.15 ± 0.02 [4]
2	Raw black beans + casitone [5]	− 1.1 ± 0.22 [4,6]	0.56 ± 0.09 [4]	0.16 ± 0.01 [4]
3	Raw black beans + casamino acids [7]	− 1.0 ± 0.38 [3,4]	0.54 ± 0.10 [4]	0.16 ± 0.02 [4]
4	Autoclaved black beans	+ 3.0 ± 0.39	0.32 ± 0.05	0.25 ± 0.04
5	Raw red kidney beans	− 2.1 ± 0.60 [3,4]	0.34 ± 0.04	0.11 ± 0.01 [4]
6	Raw red kidney beans + casitone	− 2.0 ± 0.09 [3,4]	0.50 ± 0.14 [4]	0.12 ± 0.04 [4]
7	Autoclaved red kidney beans	+ 3.1 ± 0.34	0.37 ± 0.05	0.26 ± 0.03
8	Raw white beans	+ 0.8 ± 0.43 [4]	0.40 ± 0.07	0.20 ± 0.05
9	Raw white beans + casitone	+ 2.5 ± 0.37	0.32 ± 0.12	0.24 ± 0.04
10	Autoclaved white beans	+ 2.7 ± 0.05	0.32 ± 0.08	0.23 ± 0.04
11	Raw mottled beans	+ 0.1 ± 0.37 [4]	0.25 ± 0.04	0.24 ± 0.03
12	Raw mottled beans + casitone	+ 2.5 ± 0.42	0.51 ± 0.09 [4]	0.21 ± 0.02
13	Autoclaved mottled beans	+ 2.5 ± 0.35	0.33 ± 0.02	0.23 ± 0.04
14	Raw tapiramo beans	+ 0.4 ± 0.33 [4]	0.37 ± 0.03	0.18 ± 0.02 [4]
15	Raw tapiramo beans + casitone	+ 2.9 ± 0.40	0.49 ± 0.10	0.16 ± 0.02 [4]
16	Autoclaved tapiramo beans	+ 3.0 ± 0.44	0.41 ± 0.10	0.26 ± 0.04
17	Raw soybeans	+ 4.4 ± 0.39	0.50 ± 0.08 [4]	0.30 ± 0.05
18	Raw soybeans + casitone	+ 5.6 ± 0.46	0.57 ± 0.05 [4]	0.40 ± 0.04
19	Autoclaved soybeans	+ 5.2 ± 0.38	0.34 ± 0.03	0.37 ± 0.05
20	Stock diet (without seed meal)	+ 5.1 ± 0.24	0.35 ± 0.06	0.34 ± 0.03
21	10% casitone diet (without seed meal)	+ 2.8 ± 0.36	0.34 ± 0.04	0.30 ± 0.03
22	Protein-free diet (without seed meal)	− 2.3 ± 0.20	0.24 ± 0.07	0.16 ± 0.02

[1] All diets with the exception of nos. 20 and 22 were supplemented with 0.3% DL-methionine; the stock diet used was Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[2] SD.
[3] All animals died or were killed in moribund condition within 2 weeks.
[4] Significantly different from group fed autoclaved legume diet, $P < 0.05$, calculated from t value.
[5] Bacto-casitone (trypsin-digested casein), Difco Laboratories, Detroit.
[6] Two animals died within 2 weeks.
[7] Bacto-casamino-acids (acid-digested casein), Difco Laboratories, 0.1% D-tryptophan added.

activity. Four of the 5 kidney bean samples showed amylase inhibitor action, but mottled beans lacked this activity. It was not possible to detect amylase inhibitors in soybeans with the technique used because of the strong amylase activity present in the corresponding extracts.

In growth experiments the rats fed the black or red kidney beans with high hemagglutinating activity lost weight rapidly and did not survive with this diet for more than 2 weeks. Supplementation with 10% casein digested with acid or trypsin did not improve the performance to a significant degree but autoclaving abolished the toxic effect (table 2, diets 1–7; and table 3, diets 1–3).

The diet prepared with the non-agglutinating white, mottled, or tapiramo beans did not produce weight loss and death comparable to that of the previous experiments. Growth was very poor, however, with these diets unless supplemented with tryptic casein digest. In this case growth was similar to that observed in the rats fed the autoclaved seeds (table 2, diets 8–16; table 3, diets 4–9).

Data reported in table 3 show that food intake and nitrogen absorption was low in all animals consuming unsupplemented diets containing raw beans.

Further experiments were performed to study in more detail the possible reason for the lack of growth in rats fed the unsupplemented diet of white kidney beans containing little hemagglutinin and trypsin inhibitor activity, but which was the most active in amylase inhibition (table 1). Several experiments with soybeans were also included for comparison (table 4).

Supplementation of a white bean diet with enzymatically digested casein brought growth to near-optimal values (diet 3), while casein had no significant effect (diet 4). An acid-digested casein enhanced growth significantly only after having been

TABLE 3

Performance of rats fed various methionine-supplemented diets

No.	Diet	Wt change/day	Food intake/day	N absorbed [1]	PER [2]	Pancreas wt/body wt × 100	Spleen wt/body wt × 100	Pancreas wt/spleen wt
		g	g	%				
1	Raw black beans	− 1.2 ± 0.14 [3,4,5]	2.9 ± 0.5 [5]	13.3 ± 9.9 [5]	—	0.42 ± 0.07 [5]	0.15 ± 0.02 [5]	2.9 ± 0.81 [5]
2	Raw black beans + casitone [6]	− 0.57 ± 0.61 [4,5]	4.0 ± 1.0 [5]	47.7 ± 4.5 [5]	—	0.49 ± 0.09 [5]	0.18 ± 0.06 [5]	3.1 ± 1.3 [5]
3	Autoclaved black beans	+ 3.2 ± 0.26	11.0 ± 1.3	66.8 ± 1.9	2.8 ± 0.09	0.30 ± 0.06	0.25 ± 0.02	1.20 ± 0.23
4	Raw tapiramo beans	+ 0.05 ± 0.12 [5,7]	6.1 ± 0.7 [5]	27.1 ± 4.7 [5]	0.3 ± 0.09 [5]	0.45 ± 0.16 [5]	0.18 ± 0.05	2.6 ± 0.72 [5]
5	Raw tapiramo beans + casitone	2.6 ± 0.55	10.5 ± 0.6 [5]	31.8 ± 5.8 [5]	2.5 ± 0.40	0.39 ± 0.05 [5]	0.25 ± 0.03	1.6 ± 0.23 [5]
6	Autoclaved tapiramo beans	2.9 ± 0.21	12.8 ± 0.7	64.3 ± 6.0	2.3 ± 0.16	0.29 ± 0.01	0.23 ± 0.02	1.27 ± 0.10
7	Raw mottled beans	− 0.22 ± 0.28 [5,8]	4.1 ± 0.6 [5]	17.6 ± 6.5 [5]	—	0.37 ± 0.07	0.20 ± 0.04	1.80 ± 0.45 [5]
8	Raw mottled beans + casitone	3.4 ± 0.59	13.8 ± 1.9	53.1 ± 4.0 [5]	2.4 ± 0.15	0.46 ± 0.08 [5]	0.27 ± 0.06	1.76 ± 0.56 [5]
9	Autoclaved mottled beans	3.6 ± 0.17	13.7 ± 0.8	63.0 ± 2.1	2.8 ± 0.43	0.31 ± 0.03	0.26 ± 0.03	1.20 ± 0.19

[1] Calculated: $\frac{\text{N intake} - \text{N excreted in feces}}{\text{N intake}} \times 100$.

[2] Protein efficiency ratio.

[3] SD.

[4] All animals died or were killed in moribund condition after 10 to 14 days.

[5] Significantly different from group fed autoclaved legume diet, $P < 0.05$, calculated from t test.

[6] Bacto-casitone (trypsin-digested casein), Difco Laboratories, Detroit.

[7] One animal died after 27 days.

[8] Three animals died after 25 to 27 days.

TABLE 4

Performance of rats fed diets containing white beans or soybeans

No.	Diet	Wt change/day	Food intake/day	N absorbed [1]	PER [2]	Pancreas wt/body wt × 100	Spleen wt/body wt × 100	Pancreas wt/spleen wt
		g	*g*	*%*				
1	Raw white beans	+ 0.05 ± 0.21 [3,4]	3.8 ± 1.10 [4]	42.2 ± 5.07 [4]	0.40 ± 0.14 [4]	0.31 ± 0.005 [4]	0.16 ± 0.04	1.73 ± 0.07 [4]
2	Raw white beans without methionine	− 0.32 ± 0.12 [4]	3.0 ± 0.46 [4]	35.8 ± 5.99 [4]	—	0.30 ± 0.03	0.10 ± 0.01 [4]	1.52 ± 0.17 [4]
3	Raw white beans + casitone [5]	+ 3.2 ± 0.39	11.5 ± 0.89	51.7 ± 8.54 [4]	2.8 ± 0.21	0.37 ± 0.09 [4]	0.24 ± 0.02	1.55 ± 0.39 [4]
4	Raw white beans + casein	+ 0.79 ± 0.42 [4]	5.5 ± 0.84 [4]	50.4 ± 2.87 [4]	1.3 ± 0.55 [4]	0.48 ± 0.55 [4]	0.20 ± 0.03	2.43 ± 0.42 [4]
5	Raw white beans + casamino acids [6]	− 0.64 ± 0.05 [4]	7.1 ± 0.59 [4]	—	—	0.36 ± 0.04 [4]	0.26 ± 0.06	1.38 ± 0.45 [4]
6	Raw white beans + casamino acids + tryptophan	+ 1.93 ± 0.63 [4,7]	10.2 ± 1.65	—	1.9 ± 0.62	0.42 ± 0.07 [4]	0.30 ± 0.56	1.40 ± 0.72 [4]
7	Raw white beans + glucose	− 0.25 ± 0.24 [4]	4.7 ± 0.72 [4]	47.7 ± 7.72 [4]	—	0.38 ± 0.05 [4]	0.20 ± 0.02	1.83 ± 0.22 [4]
8	Raw white beans + Na glutamate	− 0.08 ± 0.10 [4]	5.5 ± 1.10 [4]	32.2 ± 9.34	—	0.35 ± 0.05 [4]	0.17 ± 0.04	1.95 ± 0.71 [4]
9	Autoclaved white beans	+ 2.8 ± 0.40	11.0 ± 0.93	71.7 ± 2.26	2.6 ± 0.23	0.20 ± 0.02	0.20 ± 0.10	1.0 ± 0.16
10	Autoclaved white beans without methionine	+ 0.87 ± 0.15 [4,7]	6.9 ± 0.91 [4]	68.7 ± 3.86	1.3 ± 0.77 [4]	0.30 ± 0.03	0.20 ± 0.01	1.5 ± 0.17 [4]
11	Raw soybeans	+ 3.96 ± 0.52 [4]	12.3 ± 2.04	72.1 ± 3.05 [4]	3.1 ± 0.59 [4]	0.53 ± 0.08 [4]	0.27 ± 0.05	1.94 ± 0.24 [4]
12	Raw soybeans without methionine	+ 2.86 ± 0.37 [4,7]	11.3 ± 1.11	73.1 ± 2.26	2.5 ± 0.18 [4]	0.43 ± 0.07 [4]	0.21 ± 0.02 [4]	2.07 ± 0.49 [4]
13	Autoclaved soybeans	+ 5.15 ± 1.24	12.9 ± 1.87	77.3 ± 2.40	4.0 ± 0.34	0.36 ± 0.02	0.29 ± 0.03	1.24 ± 0.10
14	Autoclaved soybeans without methionine	+ 4.98 ± 0.90 [8]	12.8 ± 1.66	78.7 ± 1.63	3.8 ± 0.37	0.34 ± 0.03	0.27 ± 0.03	1.27 ± 0.23

[1] Calculated: $\frac{\text{N intake} - \text{N excreted in feces}}{\text{N intake}} \times 100$.

[2] Protein efficiency ratio.

[3] SD.

[4] Significantly different from previous group fed autoclaved legume diet, $P < 0.05$, calculated from t value.

[5] Bacto-casitone (trypsin-digested casein), Difco Laboratories, Detroit.

[6] Bacto-casamino-acids (acid-digested casein), Difco Laboratories.

[7] Significantly different from previous group, $P < 0.05$.

[8] Significantly different from no. 12, $P < 0.05$.

supplemented with tryptophan (diets 5 and 6). Glutamate did not improve weight gain of rats receiving a white bean diet, although it improved food intake (diet 8). Substitution of glucose for starch did not affect growth performance (diet 7).

Only a slight difference in weight gain was observable between the groups of rats receiving the crude white bean diets with or without methionine (diets 1 and 2) in contrast with those fed the autoclaved beans whether supplemented or not with this amino acid (diets 9 and 10). With soybean diets the effect of a methionine supplement was most pronounced when the crude seed meal was used (diets 11 and 14).

Pancreas hypertrophy was most conspicuous in rats fed raw soybeans or black beans. The diets containing raw red kidney beans, mottled beans, tapiramo beans (tables 2 and 3), or white beans (table 4), also stimulated pancreas growth.

Spleen weights significantly lower than those of the corresponding controls were observed in the rats fed raw black, red, or tapiramo beans. Moreover, low spleen weights developed when a protein-free diet was fed (table 2, diet 22). In two other groups, namely, those receiving the diets of crude white beans and cooked soybeans, respectively, both without added methionine (table 4, diets 2 and 12), significantly low spleen weights were observed.

The ratio of pancreas weight to body weight is characterized by a large standard deviation. For this reason we have included in tables 2, 3 and 4, the ratios between pancreas and spleen weights which show less variation from the mean value.

DISCUSSION

The results indicate that the 2 seed samples of black and red kidney beans containing hemagglutinin were much more toxic than the 3 samples of the same species devoid of significant hemagglutinating activity. This difference was shown by the weight changes of the respective experimental animals as well as by the mortality rates reported in tables 2 and 3. A casein digest did not overcome this toxic action in the diets prepared with the first 2 samples but produced nearly normal growth when added to rations prepared with any of the latter three; this is further evidence of an important difference in the nutritional properties of these groups of bean varieties.

Rats fed raw black beans showed the largest loss of fecal nitrogen of all groups studied. Increased fecal nitrogen excretion has been observed in animals with pancreatic hypertrophy and has been related to increased excretion of endogenous nitrogen (5). In the present experiments the pancreas of the rats consuming crude soybeans was as enlarged as that in the animals fed black beans, but fecal nitrogen excretion was much lower in the former. Reduced intestinal absorption would also result in a larger nitrogen excretion. In previously reported experiments we observed that a black bean diet, or isolated black bean hemagglutinin (Phaseolotoxin), interfered significantly with intestinal absorption (2, 6). The failure of tryptic, or tryptophan-supplemented, acid-digested casein to stimulate growth when added to black or red bean diets, could be explained by the existence of an absorption defect.

The present results are in accord with the hypothesis that oral toxicity of kidney bean agglutinin may be caused by interference with intestinal absorption (2). Kakade and Evans (7) recently observed reduction in the absorption of amino acids by rats fed navy beans and consider the interference of the hemagglutinin as a possible explanation.

A protein exhibiting a toxic effect when ingested by the oral route should be able to resist digestion in the gastrointestinal tract. To study this aspect, feces of rats (tables 3 and 4) were assayed for hemagglutinin activity. In those of all the animals fed the crude black bean diet definite blood-agglutinating activity was detected, indicating that at least part of the agglutinin had not been inactivated by digestion. No fecal excretion of agglutinating activity was observed in any of the rats fed raw soybeans. The parenteral toxicity of soybean agglutinin is similar to that of kidney beans (8) but the oral toxicity is much lower. It would be of interest to explore further whether the difference in oral toxicity between raw soybeans and kidney beans

is related to the difference in resistance to digestion of the respective agglutinins.

Stead et al. (9) and Kakade and Evans (10) have presented evidence for a partial separation of the hemagglutinating activity from the toxic activities in bean extracts. This is not necessarily in contradiction with the view that agglutinins may exhibit toxic action, because more than one hemagglutinin can be present in beans (11), and these fractions vary in their relative potencies as hemagglutinins as compared with their toxicity (12). Our previous observation that absorption of an extract from black kidney beans with stroma from human red blood cells reduces both the agglutinating and the toxic actions simultaneously (13) is in accord with this explanation. Nevertheless, the possibility of the existence of another toxic factor can not be ruled out.

The presence of a toxic hemagglutinin can not explain the poor growth-promoting capacity of diets containing white, mottled, or tapiramo beans which are devoid of this factor (table 1). Very little trypsin inhibitor activity could be found in white beans; nevertheless the growth of rats fed this material was not better than with diets made with tapiramo or mottled beans, which contain about 10 times more antitrypsin. Nitrogen absorption was low in the experiments with all 3 seeds; this is probably not due to an absorption defect, because addition of enzyme-digested casein permitted normal growth; acid-digested casein had no similar effect unless supplemented with tryptophan, an amino acid destroyed by acid treatment, but which must be present in adequate amounts in the bean seeds, because the rats did not require this supplement for growth when fed the autoclaved bean diet. This observation implies that tryptophan and probably also the other amino acids are not as available from the raw beans as from the properly heated seeds. That undigested casein, when added to a diet containing raw white bean meal did not allow for full growth (table 4, diet 4) may be an indication that the proteins from raw beans are not only poorly digested but also inhibit digestion of other nutritional proteins.

Unpublished studies in vitro showed that kidney bean proteins are very resistant to digestion and exhibit an inhibitory action on various proteolytic enzymes even after removal of the trypsin inhibitors.

The growth-preventing mechanism observed in rats receiving non-hemagglutinating white, mottled, or tapiramo beans may be present in agglutinin-containing black and red beans too, as these still show some growth-depressing action after repeated extractions with saline until the soluble hemagglutinins have been eliminated (2).

Two additional experiments were performed to rule out other possible explanations for the poor growth-promoting action of white beans. The effect of a supplement of sodium glutamate was investigated because food consumption was low in all rats fed bean diets and lack of palatibility has been cited as a possible explanation for the low growth rate (14). The result of the experiment of table 4, diet 8, does not support this view as no growth improvement was observed although food consumption was enhanced.

An amylase inhibitor was observed in beans by Bowman (15) but has not attracted the attention of the investigators concerned with the explanation of the low nutritional value of raw legumes. The action of this factor or factors was observed not only in the in vitro experiments (table 1) but also in some of the rats receiving the white bean or tapiramo bean diets causing the production of copious light colored feces; the presence of undigested starch could be detected easily by reaction with iodine solution. At autopsy these animals had bloated, white intestines similar to those described under conditions of refection in rats having ingested large amounts of raw potato starch (16). That mottled bean diets free of this amylase inhibitor did not allow better growth than white beans (table 2 and 3) and that substitution of glucose for starch in the experimental diet was without benefical effect (table 4, diet 7) would rule out the amylase inhibitor as a major factor to explain the anti-nutritional effect in the present experiments.

Pancreas hypertrophy has been observed in animals fed trypsin inhibitor containing

legumes (5). The relation between the ingestion of this enzyme inhibitor and excessive pancreas weight is accepted by most investigators in this field (17) but has not remained unquestioned (18). In the present experiments a hypertrophic pancreas was observed in all animals fed bean-containing diets, and all the legume samples had some trypsin inhibitor action. Crude soybeans and black beans were the most active in stimulating pancreas growth, although mottled beans had the highest trypsin-inhibiting activity in vitro. The white beans caused a moderate but significant increase of pancreas weight, but had little antitryptic action in vitro, whereas they were most active in amylase inhibition. No seed sample was found to be free of antitrypsin and rich in anti-amylase which could have helped to clarify the possible action of both enzyme inhibitors on pancreas growth.

The great variation in biochemical activities, both in vitro and in vivo, existing between different varieties of kidney beans as shown by the present results, should be taken as a demonstration of the importance of a clear definition of the factors present in a given legume sample for the correct interpretation of growth effects on experimental animals. These results also point to the feasibility of selecting legume varieties for low or high agglutinin or enzyme inhibitor content.

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Relation of Tissue Electrolyte Losses to the Relative Polydipsia of Early Starvation in Rats

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ABSTRACT For the first one or two days of food deprivation with water allowed, rats drink greatly in excess of apparent need. To examine the mechanism of this relative polydipsia Na, K, N and H_2O losses from tissues were determined for rats after 1 or 2 days' deprivation of food only or of both food and water. Tissue losses were estimated by material balances, taking account of changes in gut contents. Tissue losses of K were 3 to 11 times higher than tissue losses of Na on the first day of deprivation of food only. On the second day of deprivation K loss fell and Na loss rose, giving approximate equality of losses. In deprivation of food only, with water allowed ad libitum, the total tissue water loss estimated from water balance agreed closely with the sum of extracellular fluid and intracellular fluid losses calculated from Na and K losses. Disproportion of Na and K loss also occurred with food and water deprivation, after two different preceding diets, with Na or K supplements given during food deprivation and with males and females. Some of these additional treatments produced quantitative differences in the effect. These results imply that a disproportionate loss of intracellular fluid, previously reported at a later stage of starvation, appears largely on the first day, coincides with the polyuria and relative polydipsia that occur at this time but is not caused by the polydipsia. The relative decrease in size of the intracellular fluid compartment may be a cause of the relative polydipsia.

In the early stages of food deprivation, rats allowed water drink amounts in excess of apparent requirement (1). The requirement for ingested water decreases because urinary solutes are reduced and water is made available from diminishing gut contents. The water actually ingested does not decrease correspondingly and there is, therefore, a polydipsia relative to need (1). The reason for this excessive intake is not known. It might be related to changes in concentration and size of the fluid compartments. After more prolonged deprivation (3–6 days) the concentrations of Na and K in extracellular and intracellular water are not markedly different from the concentrations in normal (fed) rats (2). However, during this period, the size of the intracellular fluid compartment decreases more rapidly than does the size of the extracellular compartment (2, 3).

The observed distortion of the fluid compartments (2, 3) might occur predominantly during the period of excessive water intake. This possibility is examined in the following report. The increments (losses) of tissue electrolytes and of tissue water on successive days of deprivation were examined by metabolic balance studies which took account of changes in gut contents.

METHODS

Male and female rats of the Sprague-Dawley strain were housed in Lucite metabolism cages and were fed either powdered laboratory stock ration [1] or diet C-21, a semipurified diet (4) containing 21% casein (table 1), for at least 5 days preceding a 2-day period of either food deprivation or food and water deprivation. In experiments involving deprivation of food only, distilled water was given ad libitum at all times except in 2 experiments in which 0.1 M NaCl or 0.1 M KCl was allowed during the deprivation period. Body weights and food and water intakes were recorded daily. In one experiment, spillage from water bottles was collected in removable outer jackets surrounding the drinking spouts and was measured.

Urine and feces were separated in the metabolism cages; urine was collected in lightly stoppered graduated cylinders and the volume and refractive index (R.I.) were measured every 24 hours. Cage wash-

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[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 1
Electrolyte, nitrogen and moisture content of diets

	Diet C-21	Stock ration
Na, mEq/g	0.06	0.15
K, mEq/g	0.12	0.25
N, mg/g	30.4	37.0
H_2O, %	5	5

ings were added to the urine collections. Urinary N was determined by the Kjeldahl method and urinary Na and K by flame photometry. Wet and dry weights of feces from individual rats were measured daily; for each 24-hour collection period the dried feces from all rats in a single experiment were pooled for analysis. Fecal N was determined by the Kjeldahl method. Aliquots of the dried pooled feces were digested with H_2SO_4 and HNO_3, and Na and K in the digest solution were determined by flame photometry.

Evaporated water. Total evaporated water was measured during control and deprivation periods in 11 male rats housed singly in a metabolism cage which was completely sealed except for an air inlet and outlet. Dry air was passed continuously through the cage at about 1 liter/minute and the evaporated water was collected by absorption in a column of $Mg(ClO_4)_2$. Four of these rats were deprived of both food and water. The remainder were deprived of food with water allowed ad libitum or with water restricted to one-third to one-half of control (fed) intake.

The difference in evaporated water between fed and food-deprived states ($Ev_f - Ev_i$) was related to the difference in water intake, ($F_f - F_i$), where Ev_f and F_f refer to values found for either day 1 or day 2 of deprivation and Ev_i and F_i refer to average values found for the 2 control (fed) days (fig. 1).

Deprivation day 1 – average control):

$$(Ev_f - Ev_i) = (0.16 \pm 0.01)(F_f - F_i) + 1.03 \pm 0.14 \quad (1)$$

(Deprivation day 2 – average control):

$$(Ev_f - Ev_i) = (0.19 \pm 0.02)(F_f - F_i) + 0.18 \pm 0.30 \quad (2)$$

The regression coefficients of equations 1 and 2 were both highly significant ($P < 0.001$) and were not significantly different from each other. The displacement between the lines for day 1 and day 2 was significant ($P < 0.025$).

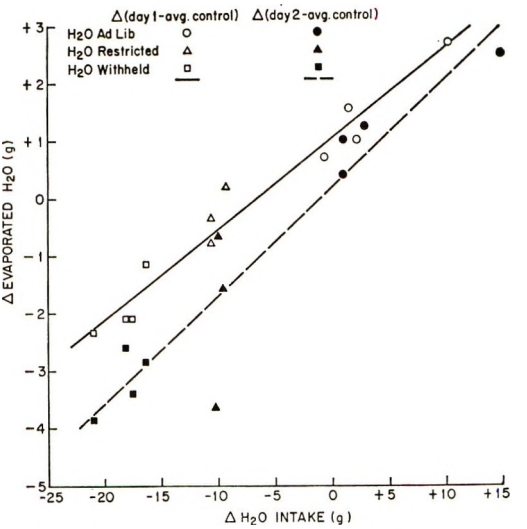

Fig. 1 Relationship between the changes induced by food deprivation, Δ(deprived-fed), in water intake and in total evaporated water at different levels of permitted water intake.

Analysis of gut contents. Male rats of the Sprague-Dawley strain of approximately comparable body weight were fed either laboratory stock ration (15 rats) or diet C-21 (27 rats) for at least 5 days. Five rats from each group were killed and the remaining rats were killed after 1 or 2 days of either food deprivation or food and water deprivation. The stomach and cecum of these rats were slit and the contents removed by eversion of the viscus. The contents of the small intestine and colon were mechanically extruded. The total gut contents for each rat were weighed and analyzed for N, Na and K. For the fed or totally deprived rats, H_2O of gut contents was also determined. For the rats deprived of food only, the H_2O of gut contents was assumed to be 80% of the total contents (6).

Calculation of tissue increments. (a) *Changes in tissue electrolytes and N.* The loss of tissue electrolytes and N during a 24-hour period of deprivation can be derived for rats in the metabolic cages from the following equations (for example, for Na):

$$\Delta B_{Na} = I_{Na} - U_{Na} - Fe_{Na} \quad (3)$$

$$\Delta B_{Na} = \Delta T_{Na} + \Delta G_{Na} \quad (4)$$

$$\Delta G_{Na} = (G_{Na})_f - (G_{Na})_i \quad (5)$$

where ΔB_{Na} is the total change in body Na, I_{Na} is intake of Na, U_{Na} and Fe_{Na} are the amounts of Na excreted in urine and feces, respectively, ΔT_{Na} and ΔG_{Na} are changes in Na of tissue and gut contents, respectively, and $(G_{Na})_f$ and $(G_{Na})_i$ are the final and initial amounts of Na in the gut, that is, at the end and the beginning of the 24-hour period of food deprivation. These equations can be combined to give the loss in tissue Na as

$$-\Delta T_{Na} = -I_{Na} + U_{Na} - [(G_{Na})_i - (G_{Na})_f - Fe_{Na}] \quad (6)$$

Thus, in the absence of ingested Na, tissue loss of Na consists of urine loss of Na less the amount of Na from gut contents which has migrated through the tissue. Tissue losses of K and N can be derived similarly.

(b) *Changes in tissue H_2O.* The total water balance equation is given as equation 7:

$$(\Delta W_f - \Delta W_i) = (F_f - F_i) + (M_f - M_i) + (Ox_f - Ox_i) - (U_f - U_i) - (Fe_f - Fe_i) - (Ev_f - Ev_i) \quad (7)$$

where ΔW, F, M, Ox, U, Fe and Ev are, respectively, the total body water increment, fluid intake, food moisture, metabolic water, urine water, fecal water and evaporated water for any two successive periods, final (f) and initial (i). The water balance is calculated in terms of difference between successive 24-hour periods to eliminate errors of measurement common to all 24-hour periods. By substitution of measured or estimated values, ΔW_f can be calculated for the first or second deprivation day. Where the initial period (i) was the pre-deprivation period, ΔW_i was estimated as two-thirds the average daily body weight increment for the 2-day fed period (5); F_i, M_i, and so on, were average values for that period. Metabolic water (Ox) was calculated assuming the pattern of energy expenditure found previously for 2-day deprivation of food (6). Urine water in grams (U) was calculated from the volume and R.I. of the urine (7). Fecal water (Fe) was estimated as twice the dry weight of feces (1).

Values for evaporated water, $(Ev_f - Ev_i)$ calculated from equation 1, were substituted in equation 7 to obtain ΔW_f for the first day of deprivation. The difference between the values calculated from equations 1 and 2 for each rat gave the change in Ev from day 1 to day 2 and was used in equation 7 to obtain ΔW_f for the second day of deprivation. The apparent variation of evaporated water difference with intake difference (fig. 1 and equations 1 and 2) may represent a variation in water evaporated from the rat or in water evaporated from the water bottle. (The latter would be expected to be higher the greater the water intake, on the assumption that the evaporative loss involved in lapping water is constant "per lap.") However, algebraically, $(\Delta W_f - \Delta W_i)$, calculated from equation 7, has the same value independently of the physical origin of $(Ev_f - Ev_i)$.

The total body water increment, ΔW_f, calculated from equation 7 for each of the periods of deprivation, consists of 2 components, the tissue water change, ΔT_{H_2O}, and the change in water of gut contents, ΔG_{H_2O}:

$$\Delta W_f = \Delta T_{H_2O} + \Delta G_{H_2O} \quad (8)$$

Therefore,

$$-\Delta T_{H_2O} = -\Delta W_f - [(G_{H_2O})_i - (G_{H_2O})_f] \quad (9)$$

where $-\Delta T_{H_2O}$ is tissue water loss and $(G_{H_2O})_i$ and $(G_{H_2O})_f$ are the amounts of H_2O in the gut at the beginning and end of the 24-hour period of deprivation.

RESULTS

The rats deprived only of food showed the polyuria and excessive water intake demonstrated previously (1). This is shown in table 2 and, for some individual rats, from the Δ intake values in figure 1.

Tissue losses of Na, K and N. Tissue losses of Na, K and N, calculated by equation 6 from urinary losses and changes in gut contents, are shown in table 3 for the various experimental groups. On the first day of food deprivation, tissue loss of K was always higher than tissue loss of Na (table 3). The ratios of K/Na loss were 1.4

TABLE 2

Water intake and urinary output of control (fed) rats and rats deprived of food

Exp. no.	Fluid allowed	Experimental group	No. of rats	H_2O intake			Urinary output		
				Control	Deprivation day 1	Deprivation day 2	Control	Deprivation day 1	Deprivation day 2
				g	*g*	*g*	*g*	*g*	*g*
1	H_2O	diet C-21 ♂	6	17.6 ± 1.6[1]	16.5 ± 2.8	16.3 ± 2.3	4.4 ± 0.8	14.5 ± 2.6	14.2 ± 2.5
2	H_2O	diet C-21 ♀	8	22.0 ± 2.0	25.6 ± 2.2	25.1 ± 2.3	7.8 ± 1.5	19.0 ± 2.1	17.5 ± 2.3
3	H_2O	stock diet ♂	7	37.0 ± 3.3	34.9 ± 6.8	30.1 ± 5.0	11.3 ± 2.4	35.4 ± 6.6	25.0 ± 4.7
4	H_2O	stock diet ♀	8	27.2 ± 1.3	20.6 ± 1.8	15.4 ± 2.0	8.8 ± 1.0	19.2 ± 1.4	10.7 ± 1.8
5	0.1 M NaCl	stock diet ♂	4	33.8 ± 1.4	83.0 ± 13.1	54.5 ± 12.8	8.5 ± 1.0	72.1 ± 6.4	44.8 + 11.2
6	0.1 M KCl	stock diet ♂	3	32.5 ± 3.8	26.7 ± 7.5	23.7 ± 5.5	10.1 ± 2.7	27.4 ± 7.1	17.0 ± 4.9

[1] Mean ± SE.

to 11.0 (when the increments were of the same sign). On the second day of food deprivation, tissue loss of K decreased and tissue loss of Na increased leading, for most groups, to approximate equality of Na and K losses.

These relationships and directions of change of Na and K loss were noted after either preceding diet and in both males and females. There were quantitative variations in the extent of change. In rats deprived of food and water the increase in Na loss from day 1 to day 2 occurred but was not statistically significant (exps. 7 and 8, table 3). In the groups allowed solutions of NaCl or KCl as drinking fluid during food deprivation the increase in Na loss from day 1 to day 2 occurred but was not statistically significant and the group allowed NaCl showed a net gain in tissue Na on the first day (exps. 5 and 6, table 3). In the group allowed KCl as drinking fluid the fall in tissue loss of K from day 1 to day 2 occurred but was not statistically significant (exp. 6, table 3).

Tissue losses of Na, K and N were greater after the stock diet than after diet C-21. Beyond this, tissue loss of N showed no consistent relationship with loss of Na or of K and showed no consistent trend from day 1 to day 2 of deprivation (table 3).

Tissue losses of water. Tissue losses of water, calculated from water balance by equation 9, are shown in table 4. Extracellular and intracellular water losses were calculated from tissue losses of Na and K (table 3), assuming that concentrations of Na and K in extra- and intracellular water, respectively, remain constant during the deprivation period (2). The sum of the losses from these 2 compartments is compared, in table 4, with the tissue water loss estimated directly.

For most groups there was no significant difference between the total tissue water losses estimated by the 2 methods. For rats allowed NaCl during the deprivation period (exp. 5, table 4) and in 2 groups of females (exps. 2 and 8, table 4) water loss estimated directly was significantly greater than that calculated from the Na and K losses.

TABLE 3

Losses of Na, K and N from rat tissues on days 1 and 2 of food deprivation [1]

Exp. no.	Fluid allowed	Diet [2]	Sex	Body wt [2]	No. of rats		Na			K			N		
							Day 1	Day 2	P value [3]	Day 1	Day 2	P value [3]	Day 1	Day 2	P value [3]
				g			*mEq*	*mEq*		*mEq*	*mEq*		*mg*	*mg*	
1	H_2O	C-21	♂	205	6	Excreted in urine	0.29 ± 0.05 [4]	0.53 ± 0.03	<0.025	0.90 ± 0.03	0.34 ± 0.02	<0.001	113 ± 10	113 ± 8	>0.5
						Migrating from gut	0.15 ± 0.02	0.01 ± 0.01	—	0.08 ± 0.01	−0.01 ± 0.03	—	24 ± 3	−6 ± 3	—
						Net loss from tissue	0.14 ± 0.05	0.52 ± 0.03	<0.005	0.83 ± 0.03	0.35 ± 0.02	<0.001	88 ± 9	119 ± 10	<0.025
2	H_2O	C-21	♀	209	8	Excreted in urine	0.16 ± 0.02	0.34 ± 0.03	<0.005	0.62 ± 0.05	0.25 ± 0.05	<0.005	135 ± 7	124 ± 4	>0.2
						Migrating from gut	0.10 ± 0.01	0.00 ± 0.01	—	0.07 ± 0.004	−0.01 ± 0.01	—	20 ± 2	−9 ± 4	—
						Net loss from tissue	0.05 ± 0.02	0.34 ± 0.03	<0.001	0.55 ± 0.05	0.26 ± 0.05	<0.005	114 ± 8	133 ± 5	>0.1
3	H_2O	stock	♂	256	7	Excreted in urine	0.84 ± 0.13	0.55 ± 0.06	<0.05	1.48 ± 0.12	0.61 ± 0.04	<0.001	187 ± 11	156 ± 10	<0.05
						Migrating from gut [5]	0.59 ± 0.04	−0.11	—	0.19 ± 0.01	−0.05	—	22 ± 2	−11	—
						Net loss from tissue	0.25 ± 0.10	0.66 ± 0.06	<0.005	1.29 ± 0.12	0.66 ± 0.04	<0.005	164 ± 9	167 ± 10	>0.5
4	H_2O	stock	♀	192	8	Excreted in urine	0.58 ± 0.03	0.33 ± 0.02	<0.001	0.89 ± 0.07	0.41 ± 0.03	<0.001	152 ± 5	121 ± 5	<0.005
						Migrating from gut [6]	0.33 ± 0.01	−0.11	—	0.11 ± 0.004	−0.05	—	10 ± 1	−11	—
						Net loss from tissue	0.25 ± 0.03	0.44 ± 0.02	<0.005	0.79 ± 0.07	0.46 ± 0.03	<0.005	141 ± 5	132 ± 5	>0.2
5	0.1 M NaCl	stock	♂	247	4	Excreted in urine	7.18 ± 0.61	5.33 ± 1.12	>0.1	1.66 ± 0.10	0.48 ± 0.02	<0.005	160 ± 12	141 ± 5	>0.2
						Migrating from gut [5]	0.51 ± 0.03	−0.11	—	0.16 ± 0.01	−0.05	—	18 ± 2	−11	—
						In fluid intake	8.30 ± 1.31	5.45 ± 1.28	>0.05	0	0	—	0	0	—
						Net loss from tissue	−1.64 ± 0.76	−0.01 ± 0.16	>0.1	1.50 ± 0.10	0.52 ± 0.02	<0.005	142 ± 12	152 ± 5	>0.5
6	0.1 M KCl	stock	♂	251	3	Excreted in urine	0.86 ± 0.23	0.38 ± 0.03	>0.1	3.74 ± 1.01	2.71 ± 0.52	>0.1	206 ± 24	158 ± 16	>0.05
						Migrating from gut [5]	0.58 ± 0.04	−0.11	—	0.18 ± 0.01	−0.05	—	22 ± 2	−11	—
						In fluid intake	0	0	—	2.67 ± 0.75	2.37 ± 0.55	>0.4	0	0	—
						Net loss from tissue	0.28 ± 0.20	0.49 ± 0.03	>0.2	0.88 ± 0.25	0.39 ±0.09	>0.1	184 ± 23	169 ± 16	>0.2
7	None	C-21	♂	252	4	Excreted in urine	0.49 ± 0.11	0.68 ± 0.04	>0.2	1.06 ± 0.06	0.47 ± 0.04	<0.001	137 ± 10	125 ± 4	>0.2
						Migrating from gut	0.15 ± 0.02	0.00 ± 0.003	—	0.05 ± 0.01	−0.01 ± 0.001	—	18 ± 2	−5 ± 1	—
						Net loss from tissue	0.35 ± 0.10	0.68 ± 0.04	>0.05	1.00 ± 0.07	0.48 ± 0.04	<0.001	119 ± 8	130 ± 4	>0.2
8	None	C-21	♀	241	8	Excreted in urine	0.45 ± 0.06	0.42 ± 0.05	>0.5	0.60 ± 0.04	0.39 ± 0.03	<0.005	134 ± 7	134 ± 2	>0.5
						Migrating from gut	0.05 ± 0.02	0.00 ± 0.005	—	0.03 ± 0.01	−0.01 ± 0.003	—	2 ± 3	−12 ± 3	—
						Net loss from tissue	0.40 ± 0.06	0.42 ± 0.06	>0.5	0.57 ± 0.03	0.40 ± 0.03	<0.01	132 ± 6	146 ± 4	<0.05

[1] Net losses from tissue are equal to amounts excreted in urine less sum of amouuts migrating to tissue from gut and amounts in fluid intake.
[2] Before deprivation.
[3] Significance of difference between day 1 and day 2.
[4] Mean ± SE.
[5] Derived using Na, K and N values from pooled dried feces of "stock ration female" experiment.
[6] Derived using Na, K and N values from pooled dried feces.

TABLE 4

Tissue water losses during food deprivation, calculated both from water balance data and from electrolyte losses

Exp. no.	Fluid allowed	Experimental group	Deprivation day 1					Deprivation day 2				
			Calculated from H_2O balance [1] Total	Calculated from Na and K losses [2]			P value [3]	Calculated from H_2O balance [1] Total	Calculated from Na and K losses [2]			P value [3]
				Extracellular fluid	Intracellular fluid	Total			Extracellular fluid	Intracellular fluid	Total	
			g	*g*	*g*	*g*		*g*	*g*	*g*	*g*	
1	H_2O	diet C-21 ♂	6.7 ± 0.6 [4]	1.0 ± 0.3	4.8 ± 0.2	5.8 ± 0.3	>0.2	7.0 ± 1.1	3.6 ± 0.2	2.1 ± 0.1	5.6 ± 0.3	>0.2
2	H_2O	diet C-21 ♀	5.7 ± 0.7	0.4 ± 0.1	3.3 ± 0.3	3.6 ± 0.4	<0.05	5.2 ± 0.8	2.3 ± 0.2	1.5 ± 0.3	3.9 ± 0.4	>0.1
3	H_2O	stock diet ♂	10.9 ± 1.7	1.7 ± 0.7	7.6 ± 0.7	9.3 ± 1.4	>0.4	9.3 ± 1.6	4.6 ± 0.4	3.9 ± 0.2	8.4 ± 0.5	>0.5
4	H_2O	stock diet ♀	8.4 ± 1.0	1.7 ± 0.2	4.6 ± 0.4	6.4 ± 0.5	>0.05	6.8 ± 0.9	3.0 ± 0.1	2.7 ± 0.2	5.7 ± 0.2	>0.2
5	0.1 M NaCl	stock diet ♂	9.4 ± 5.8	−11.3 ± 5.3	8.8 ± 0.6	−2.5 ± 5.4	<0.025	11.0 ± 1.2	−0.1 ± 1.1	3.1 ± 0.1	3.0 ± 1.0	<0.05
6	0.1 M KCl	stock diet ♂	9.5 ± 0.6	1.9 ± 1.4	5.2 ± 1.5	7.1 ± 2.8	>0.4	6.5 ± 1.1	3.4 ± 0.2	2.3 ± 0.6	5.7 ± 0.7	>0.1
7	None	diet C-21 ♂	8.8 ± 0.2 [5] 8.6 ± 0.4 [6]	2.4 ± 0.7	5.9 ± 0.4	8.3 ± 0.9	>0.5 >0.5	7.6 ± 0.3 [5] 7.7 ± 0.2 [6]	4.6 ± 0.2	2.8 ± 0.3	7.4 ± 0.2	>0.5 >0.5
8	None	diet C-21 ♀	10.6 ± 0.5	2.8 ± 0.4	3.4 ± 0.2	6.1 ± 0.5	<0.001	9.0 ± 0.3	2.9 ± 0.4	2.4 ± 0.2	5.3 ± 0.3	<0.001

[1] Using equation 9; see text.

[2] Assuming extracellular fluid loss (g) $= -\Delta T_{Na}/0.145$ and intracellular fluid loss (g) $= -\Delta T_K/0.170$, where $-\Delta T_{Na}$ and $-\Delta T_K$ are tissue losses (table 3). The error introduced by ignoring extracellular K and intracellular Na in these calculations was found to be negligible.

[3] Significance of difference between total losses estimated by the 2 methods.

[4] Mean ± SE.

[5] Calculated using equations 1 and 2 to obtain $(Ev_f - Ev_i)$.

[6] Calculated using values of $(Ev_f - Ev_i)$ actually measured.

In all the experiments where there was no significant difference between the 2 estimates of total tissue water loss, the loss estimated directly from water balance data was consistently higher than the loss estimated from electrolyte losses. In this connection, where spillage from water bottles occurs and where the spillage passes into the urine, estimate of water balance is in no way affected (eq. 7). Differences in water intake and in urine volume (eq. 7) would be substantially affected only if the amount of spillage shifted systematically with experimental conditions, that is, feeding or deprivation. Spillage, by the males fed diet C-21, in the control period and the first and second days of deprivation was 0.6 ± 0.2, 0.5 ± 0.2 and 0.3 ± 0.1 g/day, respectively. These values are not significantly different.

Urinary losses. There was no consistent pattern of urinary loss of electrolytes. Thus with the C-21 diet the urinary Na doubled from day 1 to day 2, whereas with the stock diet, the urinary Na fell (exps. 1 and 3, table 3). On the first day of deprivation, losses of Na, K and N in the urine were significantly greater ($P < 0.025$) than comparable losses from tissue, with 2 exceptions (K, exp. 6, and N, exp. 8, table 3). On the second day of deprivation, losses from urine were not significantly different from losses from tissue (exceptions: Na, exps. 4 and 5, and K and N, exps. 7 and 8, table 3).

DISCUSSION

There is a relative polydipsia during the first 1 or 2 days of food deprivation in rats (1). The losses of tissue Na and K on the first day are not in the proportion of the available Na and K in the whole body (2, 8). The form of the distortion, with high K loss and low Na loss on the first day, changing toward equality of loss on the second day of food deprivation, is the same in all situations studied, although there are quantitative differences. Particularly the distortion is qualitatively independent of whether water is allowed or not, indicating that the distortion is not the result of the relative polydipsia.

The high initial loss of K and the losses of K in general are not consistently related to N loss, although, presumably a sub-compartment of the K loss is related to it. The remainder of the K loss may be associated with depletion of cell glycogen (9). The large contribution of the liver to N loss in the early stages of starvation (10) suggests that the liver may also be the predominant source of the K/Na distortion at this time (3).

It is important to note that the balance approach to this problem is feasible only if change in gut contents is specifically taken into account. The urine content of Na and K is not interpretable in this way, particularly on the first day of food deprivation when the initial gut load is large and the relative polydipsia occurs most consistently (1).

The distortion in tissue loss of Na and K implies either a distortion in concentration of electrolytes in tissue fluids or a distortion in size of fluid compartments.

Kohn (11) found no appreciable change in plasma concentration of Cl until about the fourth day of food deprivation. Na, K and Cl concentrations in extra- and intracellular fluids (by whole body and tissue analysis) were not substantially altered from normal (fed) levels after 3 to 6 days of food deprivation (2), but the early fasting period (1 to 2 days) was not examined. On the assumption that tissue concentrations of Na and K do not change, there is, in the groups fasted with water allowed, equality of tissue water loss estimated from water balance and tissue water loss estimated from tissue losses of Na and K. This agreement gives partial validation of the assumption. (The agreement does not fully validate the assumption as it is conceivable that Na and K concentrations might alter in opposite senses and still yield agreement between total water losses.) The discrepancies, in the standard food-deprived groups, between tissue water loss estimated directly and loss derived from electrolyte losses, are small and not statistically significant but are systematic. These discrepancies could arise from residual errors in the direct estimation of water balance.

In the group supplemented with Na in the drinking water and in 2 groups of fe-

males, the same form of distortion of Na and K loss from tissues occurred but the directly estimated tissue water loss differed significantly from the tissue water loss derived from electrolyte losses. In the case of the females this may be an effect of the estrous cycle (12). In the case of the group given hypotonic NaCl as drinking fluid, possible balance errors are magnified by the large volume drunk and the large amount of Na correspondingly ingested. Also an appreciable amount of the Na ingested was retained. In these groups the assumption that electrolyte concentrations are unchanged cannot be sustained.

In all other groups it is reasonable to assume that concentration of Na and K in tissue fluids does not alter. The distortion of loss of Na and K on the first day of food deprivation implies, therefore, a distortion of size of fluid compartments at this time, notably a reduction in size of the intracellular compartment. This agrees with the form of change found by whole body analysis after 3 to 6 days (2) and by measurement of thiocyanate space and total body water after 4 or 6 days of food deprivation (13). However, in our experiments, the distortion does not continue and makes up only about half the total distortion found after the longer periods. This might mean that a later similar distortion occurs that is not accompanied by a polydipsia, which ends after 2 to 4 days (1). However, the errors inherent in the various methods used (balance studies, whole body analysis, dilution estimates) may be such that the results can show similarity in direction and forms of change but do not justify detailed quantitative comparison.

The changes in Na and K loss that occur on food deprivation are associated with the polydipsia. If they cause the polydipsia then they probably do so through a decrease in volume of the intracellular compartment and not by a decrease in volume of the extracellular compartment or by osmotic changes (14). But this conclusion must be tentative until cellular and extracellular concentrations at this stage of food deprivation have been examined in greater detail.

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In vitro Uptake of Selenium-75 by Red Blood Cells from the Immature Ovine during Varying Selenium Intakes[1,2]

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ABSTRACT Six crossbred wether lambs were used for preliminary study of the effect of varying Se intake upon uptake of ^{75}Se in vitro by ovine red blood cells. In a second experiment, 16 crossbred wether lambs were equally allotted among 4 dietary Se levels of 0.014, 0.264, 0.514 and 5.014 ppm. The feeding trial lasted 104 and 107 days for experiments 1 and 2, respectively. Lambs receiving 0.264 and 0.514 ppm Se gained faster, consumed more feed and had a higher feed efficiency than lambs in other dietary groups. Lambs on 0.014 ppm Se performed better than with the 5.014 ppm level. Adequate vitamin E may have prevented deficiency syndromes in lambs fed the low level of Se. Two lambs on the 5.014 ppm level exhibited symptoms of chronic selenosis. The uptake of ^{75}Se was significantly correlated with incubation time in the presence of an O_2–CO_2 mixture. The initial influx and rate of in vitro uptake of ^{75}Se by ovine blood cells decreased with increasing Se level in the diet. The in vitro uptake of ^{75}Se by red blood cells proved to be a promising technique to assess the nutritional Se status in the immature ovine.

The present emphasis on selenium studies is based on the recognition of the dual role of this element as a toxic agent and as an essential micronutrient. Selenium toxicity in animals was recorded as early as 1856, but the element was not associated with livestock poisoning until 1933. In 1957, selenium emerged as a trace mineral of great importance in the dietary of animals.

The recent discoveries of the nutritional significance of Se provided great impetus for studies on its metabolic role and physiological relationship to other nutrients. One of the biggest impediments in Se studies has been the lack of a convenient but highly sensitive and precise technique for following selenium metabolism. However, the use of radioactive selenium in tracer quantities has partially overcome this difficulty and activation analysis provides a sensitive method for its determination.

Several studies have demonstrated the incorporation of ^{75}Se into circulating red blood cells (RBC) following its administration in several species. The radioactive element moved into ovine cells in vitro and the uptake was higher in animals receiving a Se-deficient diet (1). This suggests a possible correlation between RBC uptake of ^{75}Se and dietary levels of Se. With this hypothesis, the technique may have value to assess dietary Se intake and availability to the animal.

In view of the above, the present study was conducted to evaluate Se status of lambs fed varying Se intakes. The other criteria were performance and general appearance.

EXPERIMENTAL

Two separate experiments were conducted. In both experiments, crossbred wether lambs were used. All lambs were housed in individual cages, 85-cm square and equipped with slatted floors. Each cage was provided with a stainless steel feeding trough and a white porcelain pan for drinking water. The animals were fed and watered ad libitum. The semipurified ration was fed after mixing 2 parts of feed with one part of tap water.[3] The basal ration (tables 1 and 2) contained 0.016 and 0.014 ppm Se, respectively, for the first and second experiments as determined by activation analysis.

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[3] Selenium concentration determined by activation analysis was 0.002 ppm (General Dynamics Corporation, San Diego).

TABLE 1
Composition of basal ration

	%
Torula yeast [1]	6.5
Cellulose [2]	30.0
Cornstarch [3]	30.0
Glucose [3]	23.5
Urea [4]	3.0
Lard [5]	3.0
Mineral mixture [6]	3.6
Vitamin mixture [6]	0.4

[1] Red Star Yeast and Product Company, Milwaukee, Wisconsin.
[2] Solka Floc (BW-40), Brown Company, Berlin, New Hampshire.
[3] Cerelose, Clinton Corn Processing Company, Clinton, Iowa.
[4] John Deere Chemical Company, Tulsa, Oklahoma.
[5] Swift and Company, Kansas City, Missouri.
[6] Refer to table 2.

TABLE 2
Composition of vitamin and mineral mixtures

	% of ration
Vitamin mixture:	
Choline chloride [1]	0.10
Vitamins A and D_3 [2]	0.08
Vitamin E [3]	0.22
Mineral mixture: [4]	
$CaHPO_4$	1.71
K_2CO_3	1.10
$MgSO_4$	0.36
NaCl, iodized [5]	0.36
	mg/kg of ration
$FeSO_4 \cdot 7H_2O$	400.00
$ZnSO_4 \cdot 7H_2O$	131.00
$Na_2B_4O_7 \cdot 10H_2O$	123.00
$MnSO_4 \cdot H_2O$	90.00
$CuCO_3 \cdot Cu(OH)_2$	14.00
$NaMoO_4 \cdot 2H_2O$	2.50
$CoCl_2 \cdot 6H_2O$	0.34

[1] Merck and Company, Rahway, New Jersey.
[2] Contained 2250 and 400 IU per g mixture of vitamins A and D_3, respectively. Thompson-Hayward Chemical Company, Kansas City, Missouri.
[3] *dl-α*-tocopheryl acetate, 44.5 IU/g, NoPCO Chemical Company, Newark, New Jersey.
[4] All certified reagent grade, Fisher Chemical Company, St. Louis.
[5] Contained 0.02 of 1% KI, Barton Salt Company, Hutchinson, Kansas.

Experiment 1. Six wether lambs with an average weight of 30 kg were assigned at random to a single dietary level of Se, 5 lambs fed the semipurified diet and one lamb a natural ration. Selenium was added to the basal ration at zero, 0.5, 1.0, 1.5, and 5.0 ppm levels, as Na_2SeO_3. The natural ration consisted of: (%) cottonseed hulls, 40; ground shelled corn, 25.8; soybean meal (44%), 18; blackstrap molasses, 10; alfalfa meal, 5; iodized cobalt salt,[4] 0.7; dicalcium phosphate,[5] 0.5; and vitamin A and D_3 mixture,[6] 0.026.

Blood samples were collected from the jugular vein of each lamb initially (before Se treatment was initiated) and at biweekly intervals (during Se treatment). The samples were analyzed immediately for RBC uptake of ^{75}Se by the method of Wright and Bell (1). Fifteen-milliliter portions of freshly drawn blood were incubated with 90 $m\mu Ci$ of ^{75}Se in 100 μliters of isotonic (0.90%) saline. The radioactive selenium was obtained from Oak Ridge National Laboratory as selenous acid ($H_2{}^{75}SeO_3$) with a concentration of 8.72 mCi per ml of acid and specific activity of 58,133 mCi per g Se.

Tubes containing the blood sample and ^{75}Se were rotated once every 10 minutes during incubation. Temperature was maintained at 38 ± 1° and an O_2-CO_2 mixture [7] was passed into the tubes at the rate of 30 bubbles per minute. Two 1-ml aliquots were drawn directly from incubation tubes at a 30-minute interval throughout a 3-hour period. Gamma counting of the samples before and after washing was carried out in centrifuge tubes in a well-type scintillation counter.[8]

The blood cells were washed 3 times with 9 ml cold isotonic saline. Samples were centrifuged, with the tubes cushioned in ice water. The washings were drawn off via a suction device. Drops of saline were added to the washed cells to bring the volume to 1 ml and the sample was counted. Samples were kept in ice water between 5° and 8° at all times after incubation and before final counting.

The percentage uptake of ^{75}Se in all samples was corrected to a packed cell volume of 0.4. An average of 3 hematocrit readings at each sampling time was the basis used for packed cell volume.

Experiment 2. Sixteen lambs weighing approximately 25 kg were fed the basal ration (table 1) for 4 weeks, after which they were divided into 4 groups of 4 lambs

[4] Contained 22.44 g $CoCl_2 \cdot 6H_2O$ per 100 kg iodized salt (0.02 of 1% KI).
[5] Guaranteed to contain 18.5% P and 20 to 24% Ca. International Minerals Company, Skokie, Illinois.
[6] Refer to table 2.
[7] High pressure 95% O_2 and 5% CO_2 mixture, USP. Liquid Carbonic Division, General Dynamics Corporation, San Diego.
[8] Picker Nuclear Instrument Company, Cleveland (Cat. no. 2804).

each on the basis of weight and daily feed intake. Selenium was added to the basal ration at zero, 0.25, 0.5 and 5.00 ppm levels as Na_2SeO_3.

Blood samples were collected from each lamb after the basal ration had been fed for 4 weeks, and at weekly intervals after initiation of Se treatments. Red blood cell uptake of ^{75}Se was determined from each sample by incubation with ^{75}Se for 1 hour. The radioactive selenium used in this experiment was sodium selenite [9] ($Na_2{}^{75}SeO_3$) in sodium hydroxide solution of pH 10. The concentration was 1.07 mCi per ml and specific activity, 1338 mCi per g Se. All other measurements were made exactly as in experiment 1.

RESULTS

Performance. Feed consumption of lambs in both experiments showed great variation during the first 2 weeks of the experimental period. After the animals had been fed the experimental rations for 3 weeks, those in all dietary groups increased their feed intake and were consuming approximately equal amounts of feed. The average daily feed intake was 848 ± 46

[9] Obtained from Nuclear-Chicago Corporation, Des Plaines, Illinois.

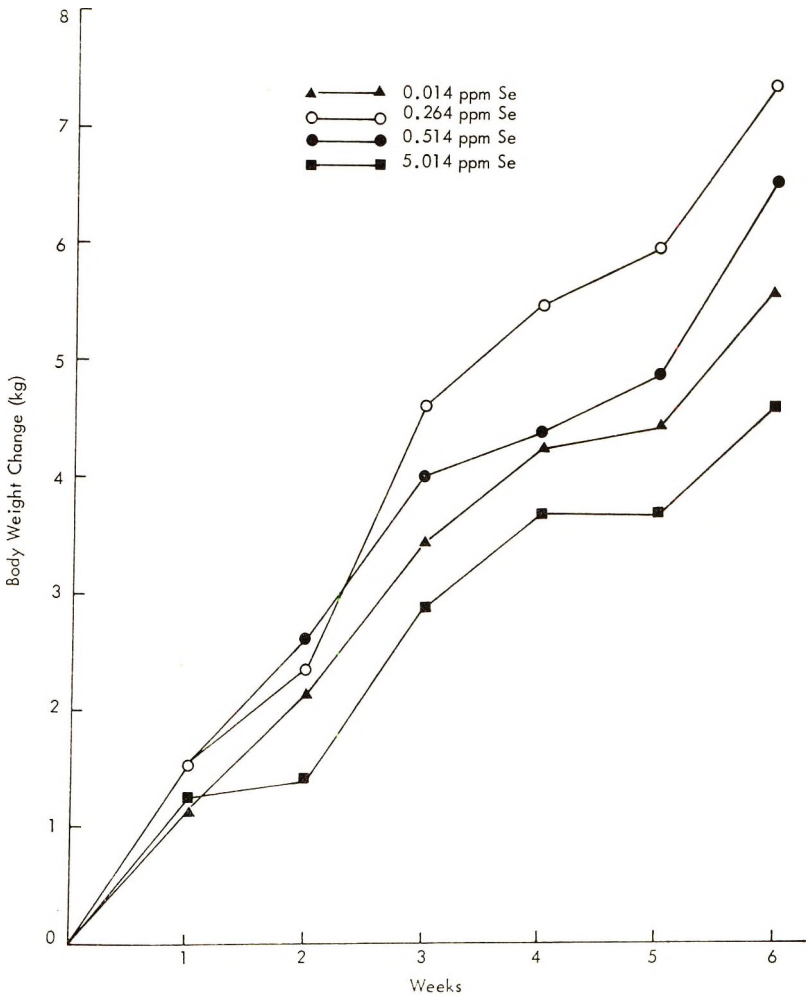

Fig. 1 Body weight change in lambs fed varying selenium intakes.

and 1320 ± 122 g per day in experiment 1 and 2, respectively; the level of Se in the ration did not consistently affect feed intake, although lambs fed 0.26 ppm Se in experiment 2 consumed somewhat more feed than lambs on the other treatments. Mixing feed with water (2:1) improved feed intake and decreased the amount of wasted feed. Free access to drinking water during feeding increased feed intake by approximately 25%.

Body weight change at weekly intervals of lambs in the second experiment is shown in figure 1. Lambs given 0.26 ppm Se gained faster than those fed the other Se levels. Two lambs receiving 5.01 ppm Se showed some symptoms of toxicity. The animals became emaciated, lost wool on the croup and were unthrifty. These symptoms were not observed in the other 2 lambs in the same dietary group. One lamb fed the basal diet died on day 87 of the experiment due to urinary calculi.

In vitro uptake of ^{75}Se by red blood cells. Exp. 1. A linear correlation existed between RBC uptake of ^{75}Se and incubation time regardles of Se level in the ration (fig. 2). The Y-intercept (initial influx) of ^{75}Se was highest for the lambs fed the basal diet, decreasing gradually with increasing Se level in the diet ($P < 0.05$). The lamb fed natural ration had an initial influx between the 1.51 and 5.01 ppm levels. There was no consistent trend in

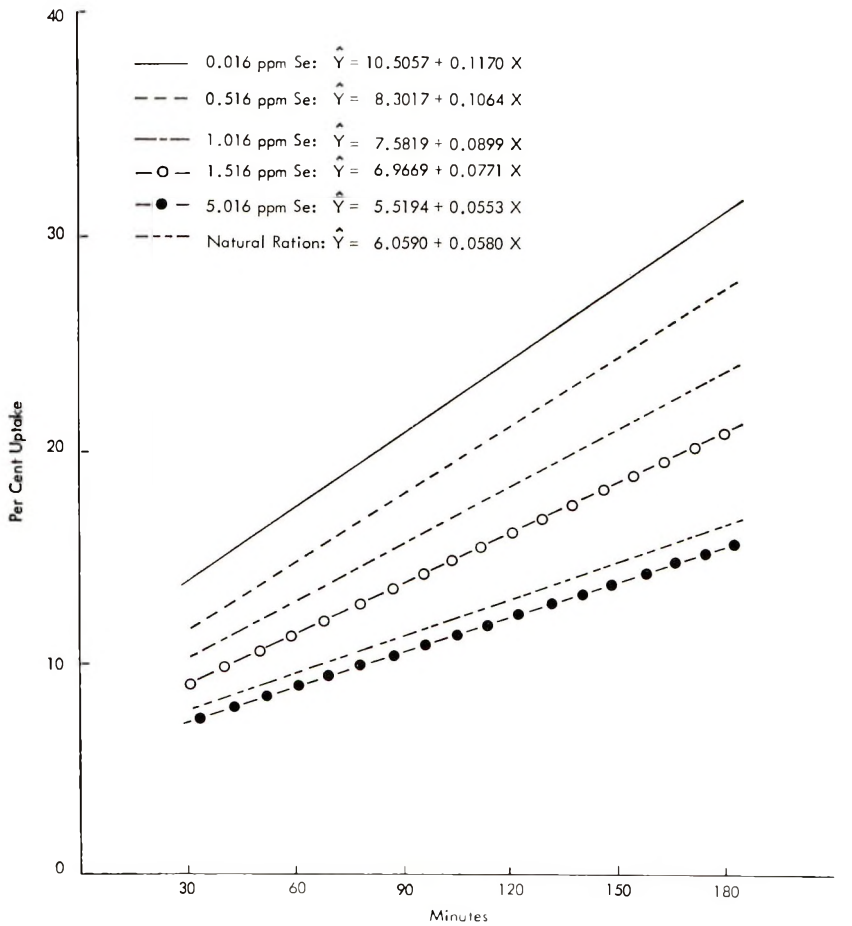

Fig. 2 Effect of varying dietary selenium levels upon in vitro uptake of ^{75}Se by ovine blood cells (exp. 1).

the initial influx of ^{75}Se with time on the ration.

Selenium level in the diet had a marked influence on the rate of uptake of ^{75}Se by ovine blood cells when incubated in an atmosphere of O_2-CO_2 mixture (fig. 2). The rate of uptake was highest for the lamb fed the basal diet, decreasing gradually with increasing Se level in the ration; that of the lamb fed a natural ration was lower than the 1.51 ppm Se but slightly higher than the 5.01 ppm level.

When experimental rations were arranged in the order of increasing Se level, the difference in the b value (regression coefficient) of any 2 succeeding treatments was not significant; however, the regression coefficients of any dietary levels not in succession differed statistically ($P < 0.05$). The b value of the natural ration differed ($P < 0.05$) from dietary levels lower than 1.51 ppm level, while the difference from 1.51 and 5.01 ppm levels was not significant. No distinct pattern was observed in the slope values characteristic of the number of weeks the lambs were on the various Se levels.

The average hematocrit value was 30%, while the average hemoglobin value was 9.1 g per 100 ml blood. Average hematocrit and hemoglobin values of lambs on 1.51 and 5.01 ppm Se were slightly higher than those of lambs on 0.01, 0.51 and 1.01 ppm levels but lower than those of the lamb fed a natural ration. There was no trend in hematocrit and hemoglobin values at biweekly intervals.

Exp. 2. The cellular uptake of ^{75}Se was dependent on dietary Se levels. Cellular uptake of ^{75}Se decreased with increasing Se level in the diet ($P < 0.05$) as shown in figure 3; there was only a slight difference in uptake between the 0.51 and 5.01 ppm levels. The percentage uptake of ^{75}Se was highest after the basal ration had been fed for 4 weeks (before varying Se treatments were initiated), with a rapid decrease in subsequent weeks (during Se treatments).

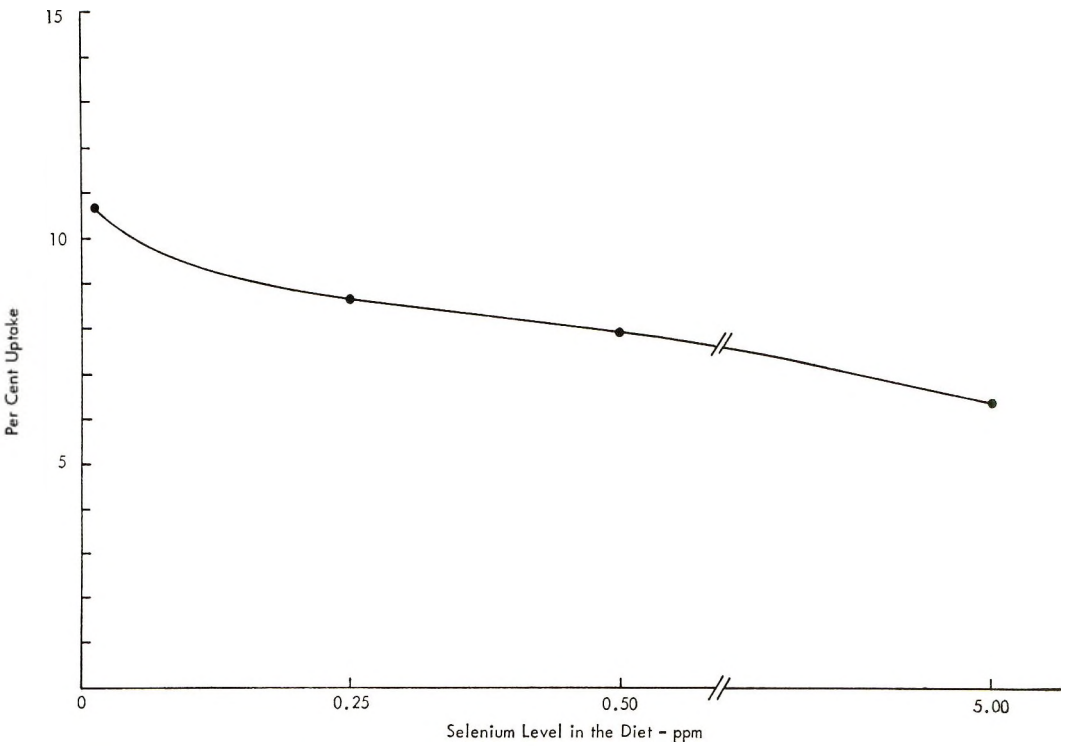

Fig. 3 In vitro uptake of ^{75}Se by red blood cells of lambs fed varying dietary selenium levels (exp. 2).

There was no apparent trend in cellular uptake of ^{75}Se at weekly intervals during the period of varying Se intake. Lambs fed basal ration (0.01 ppm Se) showed a wider variation in cellular uptake of ^{75}Se at weekly intervals than lambs in the other dietary groups.

The effect of dietary Se level on the uptake of ^{75}Se was observed after the first week of treatment. The 0.01 ppm level showed the highest uptake of ^{75}Se during the entire experiment. It differed ($P < 0.05$) from other dietary groups except for the first week, where the difference from the 0.26 ppm level was not significant. The 0.26 ppm level of Se resulted in a higher uptake than the 0.51 ppm level at all sampling periods; however, the difference was significant ($P < 0.05$) only in the third and sixth week. The uptake at the 5.01 ppm level was significantly lower ($P < 0.05$) than for any of dietary groups from the first through the sixth week of sampling.

There was considerable variation in hematocrit and hemoglobin values in the same lamb at weekly intervals or between animals in the same dietary group. When hematocrit values reached a value of less than 30%, the correction of Se uptake to 0.4 packed cell volume resulted in an abnormally high uptake value.

DISCUSSION

Performance. Body weight gain during the 12-week period of the 5 lambs used in experiment 1 was lower than that in experiment 2, which may be related to the difference in daily feed intake. An adjustment period of 4 to 6 weeks may be required for the lambs to become accustomed to the semipurified diet. Daily weight gain during the last 6 weeks of experiment 1 showed agreement with the growth data of lambs in experiment 2. Average daily gain of lambs in experiment 1 receiving 0.01, 0.51 and 5.01 ppm Se was 130, 179, and 143 g, respectively. The average daily gain of lambs in experiment 2 for the same levels of dietary Se was 140 ± 38, 161 ± 35, and 125 ± 61 g, respectively.

Definite conclusions regarding body weight gain with respect to dietary Se intake are difficult to make since they were not statistically significant, due to the wide variation in individual performance of lambs in the same dietary group. The relatively poor performance of lambs fed the basal ration is in agreement with the reports of Clark and Filmer (2) and Hartley and Grant (3) that Se-deficient hoggets were unthrifty. None of the lambs, however, exhibited deficiency symptoms other than poor gain and loss of wool in either experiment. Symptoms of white muscle disease were not observed as reported by Allaway and Cary[10] in lambs and cattle maintained without interruption with a ration containing less than 0.05 ppm of Se. Similarly, Oldfield et al. (4) reported that white muscle disease was prevalent in lambs from ewes receiving hay containing less than 0.02 ppm Se. The fact that our experimental ration contained adequate vitamin E (97 IU per kg feed) may have spared part of the Se and reduced the need for the element. Hopkins et al. (5) produced syndromes of Se deficiency in rats fed a Se-deficient diet, but no deficiency symptoms were observed when vitamin E was included in the ration.

Several studies have shown that diets containing more than 0.1 ppm Se resulted in increased weight gain of lambs and young sheep (6–9). This is in agreement with the better performance obtained with lambs on 0.26 and 0.51 ppm Se obtained in experiments 1 and 2. Oldfield et al. (4) and Andrews et al. (10), however, suggested 0.07 ppm Se as the required level for sheep. This lower requirement compared with the requirement in this study and other reports may be explained by a difference in the nature of the diet. It has been shown in earlier reports that Se in plants is generally present in an organic form and is more available to the animal as compared with inorganic Se supplements.

The observations made on lambs receiving 5.01 ppm Se are in accord with reports made by other investigators that 4 to 5 ppm Se is the lower toxic level of Se in animal diets. Blaxter (11) reported that Se administration resulted in loss of body weight in sheep kept on soil containing Se.

[10] Allaway, W. H., and E. E. Cary 1966 The environmental background of selenium deficiency diseases. Feedstuffs, 38: 62.

Similarly, Moxon et al. (12) observed poor growth in steers grazed on highly seleniferous range. Arsenic and sulfur, particularly sulfate, have been known to reduce Se toxicity. The As content [11] of the ration used in this study, however, is certainly at a minimum (0.018 ppm) to give any effect. The calculated sulfate content of ration used in this study was 82% of the total S (0.123% of ration) in the ration.

In vitro uptake of ^{75}Se by ovine blood cells. An increase in uptake of Se with time of incubation was observed regardless of treatment when cells were incubated with an O_2-CO_2 mixture (fig. 2). Wright and Bell (1) obtained similar results in ewes. They reported that increased Se uptake beyond the initial influx may indicate a mechanism requiring active respiratory processes by the cell membrane. The process may involve an oxidation of selenite to selenate; Rosenfeld and Beath (13) reported that selenate can be formed quantitatively by oxidation of selenite in $NaHCO_3$ solution.

The initial influx and rate of uptake of Se by ovine blood cells decreased with increasing Se level in the diet. The difference in the initial influx of the element was not as marked as the difference in the rate of cellular uptake between dietary Se levels. The less significant effect in the initial influx of Se between dietary levels may be explained by a limited initial O_2 concentration in the blood cells. The intercept of lambs fed the basal ration differed significantly from the 1.51 ppm Se and higher but not from the lower levels of dietary Se. This suggests that cells from a Se-deficient animal have a faster initial influx, or a lower content of Se. The latter would actually be an isotope-dilution assay for Se in the red blood cells.

The uptake of Se per unit time during incubation in an atmosphere of O_2-CO_2 mixture exhibited marked differences between dietary levels of Se. The rate of uptake decreased with increasing Se in the diet with only a slight decrease at levels above the 1.51 ppm level of Se. The limited uptake of ^{75}Se at higher dietary Se levels indicates the element is not continuously cumulative in the blood cells. The rate of uptake may be partly determined by the influence of dietary Se on the amount of Se present in the cells.

The initial influx of ^{75}Se at 2 weeks after initiation of treatment was significantly lower than the influx at 4 weeks. The rate of Se uptake at this time, however, was significantly higher than at other weeks except at 8 weeks. This suggests that an equilibrium between dietary intake, body stores and excretion was reached by 4 weeks and was maintained while on that level of Se.

Results obtained from experiment 2 when cells were incubated for 60 minutes show that in vitro uptake of ^{75}Se by ovine blood cells is a function of dietary Se levels and duration of Se intake. It is evident from the data that cellular uptake of ^{75}Se by ovine blood cells can serve to differentiate a deficient dietary Se intake by the animals.

All dietary groups decreased significantly in ^{75}Se uptake by RBC in the first week after initiation of Se treatment. Lambs receiving added Se in the ration showed no change in cellular uptake after the first week they were fed the Se diet except at the 0.51 and 5.01 ppm levels where cellular uptake decreased significantly in the sixth week. These results were not in complete accord with experiment 1 where equilibration in ^{75}Se uptake was attained between the second and fourth week. Wright and Bell (1) reported that 6 weeks were required to reach equilibrium in ewes. Studies on retention and excretion of ^{75}Se by the author [12] showed that Se equilibrium may be established at a much earlier time.

The in vitro uptake of ^{75}Se by RBC proved to be a promising technique to assess the nutritional Se status in the immature ovine. The feasibility and reproducibility of the technique are some of its advantages. Sensitivity was fair although it may be difficult to determine the difference between small dietary increments; it would definitely differentiate lambs maintained with a deficient, adequate or subtoxic level of Se. There are 3 ways in which this technique can be used: (a) determination of initial

[11] Determined by activation analysis (General Dynamics Corporation, San Diego.

[12] Lopez, P. L. 1966 Selenium metabolism in lambs fed varying selenium intakes. Ph.D. Dissertation, University of Missouri, Columbia, Missouri.

influx of ^{75}Se, (b) rate of uptake (regression coefficient) of ^{75}Se by RBC with time of incubation in the presence of an O_2-CO_2 mixture, and (c) uptake of ^{75}Se at some pre-set time of incubation in the gas mixture. One may safely assume that after 4 weeks on feed, the lambs would attain Se equilibrium in the body such that ^{75}Se uptake can be evaluated independent of time at a given level of Se intake. The first measurement can be made quickly but is the least sensitive. It can be used to differentiate an Se-deficient animal from a lamb receiving 1.5 ppm Se or higher; no significant difference would be expected between a deficient and adequate or between adequate and subtoxic levels. Determination of the regression coefficient is more sensitive. A difference of 0.5 ppm in dietary Se levels (from basal through 1.5 ppm) would give a significant difference. At dietary levels above 1.5 ppm, rate of uptake was not significantly different. The third measurement combines somewhat the relative results of the first two.

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Destruction of Rachitogenic Activity of Isolated Soybean Protein by Autoclaving as Demonstrated with Turkey Poults [1,2]

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ABSTRACT Investigations were conducted to determine the effect of increasing vitamin D_3 levels and the effect of autoclaving on the rachitogenic activity of isolated soybean protein (C-1 protein—Skidmore). One-day-old poults were fed purified diets of the glucose-C-1 protein type. The diets contained calcium, phosphorus and vitamin D_3 at levels equal to or greater than National Research Council recommendations. These results demonstrate that autoclaving destroys rachitogenic activity exhibited by C-1 protein. Autoclaving C-1 protein increased tibia ash values, and concomitantly serum alkaline phosphatase activity was decreased. However, autoclaving the C-1 protein for 60 minutes was most effective in reducing the rachitogenic effects of C-1 protein. In 2 out of 3 experiments, use of autoclaved C-1 protein in the diet also increased weight gains and serum calcium levels. In general, increasing dietary levels of vitamin D_3 lowered serum alkaline phosphatase activity. The effects of increasing vitamin D_3 levels on weight gains, bone ash and serum calcium were variable between experiments. Neither autoclaved C-1 protein nor increasing vitamin D_3 levels had any effect on serum inorganic phosphate.

There has been interest in the rachitogenic properties of isolated soybean protein [3] and means of overcoming this undesirable property. Carlson et al. (1, 2) observed rickets in turkey poults fed a semipurified diet containing C-1 protein and levels of calcium, phosphorus and vitamin D_3 adequate for optimal bone formation, according to NRC (3) standards. Autoclaving the C-1 protein for 30 minutes at 120° partially destroyed its rachitogenic activity. Increasing the vitamin D_3 level several-fold also decreased the rachitogenic activity exhibited by C-1 protein.

Jensen and Mraz (4) working with diets similar to those used by Carlson et al. (1) also reported that bone calcification was markedly improved in chicks fed autoclaved C-1 protein compared with bone calcification of chicks receiving the untreated C-1 protein.

In studies where calcification is being measured, it is a common procedure to use terminal bone ash values as a measure of calcium deposition. In some instances, it is desirable to determine the extent of calcification before the termination of an experiment. One such method, using toe bone analysis, has been developed by Migicovsky and Nielson (5). However, this analysis can be used only a limited number of times per experiment. It has been suggested that serum alkaline phosphatase activity in chicks offers another method of measuring differences and similarities among antirachitic substances (6).

The purposes of this work were: to reaffirm the effect of autoclaving C-1 protein; to define more clearly the autoclaving time necessary for the maximal reduction of the rachitogenic properties of C-1 protein; to determine the effect of a wider range of vitamin D_3 levels than used previously; and to ascertain whether there was a direct relationship between the rachitic condition of poults as measured by tibia ash with that as measured by serum alkaline phosphatase activity.

The criteria used in this study were body weight gain, tibia ash, serum alkaline phosphatase activity, serum inorganic phosphate and serum calcium. The data were subjected to analysis of variance, F and LSD tests using the procedures cited by Snedecor (7).

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[2] Approved for publication by the Director of the South Dakota Agricultural Experiment Station as Paper no. 778 of the Journal series.

[3] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

EXPERIMENTAL

In the experiments reported here, poults were fed diets containing 44% C-1 protein as shown in table 1. The levels of calcium, phosphorus and vitamin D_3 were higher than NRC (3) recommendations.

The dry C-1 protein was autoclaved by placing 5-kg batches in enamel trays and spreading them to a uniform depth of 2.5 cm. The trays were then placed in an autoclave and brought to 100° by steaming and then to 120° for the designated period of time.

One-day-old Wrolstad Small White poults were kept in electrically heated wire-floor batteries. Water and food were supplied ad libitum for the 4-week experimental periods. Nine or 10 poults were placed in each pen and 3 pens were allotted to each ration treatment. All experiments were conducted as random complete block designs with factorial arrangements.

In experiment 1, 4 levels of vitamin D_3 (880, 1760, 3520 and 7040 ICU/kg of diet) were fed with untreated C-1 protein or C-1 protein autoclaved for 40 minutes. Experiment 2 was similar except that only 3 vitamin D_3 levels were used (880, 1760 and 3520 ICU/kg of diet) and the autoclaving time was increased to 60 minutes. In experiment 3, 2 levels of vitamin D_3 (1760 and 3520 ICU/kg of diet) were fed with untreated C-1 protein or C-1 protein which had been autoclaved for 20, 40 or 60 minutes.

TABLE 1

Composition of basal diet

	%
Isolated soybean protein [1]	44.0
Glucose monohydrate [2]	42.0
Cellulose [3]	3.0
Corn oil	2.0
Dicalcium phosphate [4]	4.0
Limestone	1.5
DL-Methionine	0.7
Glycine	0.5
Minerals,[5] vitamins,[6] antibiotics [7] antioxidant [8]	2.3

[1] Assay Protein C-1 (90% protein), Skidmore Enterprises, Cincinnati.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Dicalcium Phosphate, Feed Grade, Central States Phosphate, Inc., Weeping Water, Nebraska.
[5] Added minerals in mg/kg of diet were: NaCl, 4994.0; KCl, 4994.0; $MgSO_4 \cdot 7H_2O$, 3014.0; $MnSO_4 \cdot H_2O$, 198.0; $FeSO_4 \cdot 7H_2O$, 308.0; $CuSO_4 \cdot 5H_2O$, 8.8; $ZnSO_4 \cdot H_2O$, 308.0; KI, 11.0; $Na_2MoO_4 \cdot 2H_2O$, 2.2; Na_2SeO_3, 4.4; H_3BO_3, 11.0; $AlK(SO_4)_2 \cdot 12H_2O$, 123.2; Na_2SiO_3, 50.6; NaBr, 2.2; and $CoCl_2 \cdot 6H_2O$, 22.0.
[6] Added vitamins in mg/kg of diet (except where stated otherwise) were: folic acid, 8.8; pyridoxine, 22.0; riboflavin, 22.0; thiamine, 22.0; Ca pantothenate, 44.0; niacin, 99.0; choline (25% in wheat middling carrier), 14,080.0; p-aminobenzoic acid, 110.0; ascorbic acid, 22.0; biotin, 440.0 μg/kg; vitamin B_{12} (1 mg/g), 33.0; vitamin E (275,000 IU *dl*-α-tocopheryl acetate/kg), 35.0; vitamin A palmitate (325,000 IU/g), 51.2; and menadione sodium bisulphite (11.44 mg/kg), 22.0.
[7] Oxytetracycline, 2.0 mg/kg.
[8] Ethoxyquin, 110.0 mg/kg.

At the termination of each experiment blood was taken by heart puncture from a representative sample of birds (5 when possible) from each group. The blood was pooled and kept on ice until centrifuged. After centrifugation the serum was removed, placed in vials and stored at −20°. Serum alkaline phosphatase activity was determined by the method of Bodansky (8, 9). Serum inorganic phosphate was determined by the Fiske and Subbarow method (10). Serum calcium was measured by the use of a Perkin-Elmer 303 atomic absorption spectrophotometer and tibia ash was determined by a modified AOAC (11) method.

RESULTS

Experiment 1. Table 2 shows that body weight gains, serum calcium and serum inorganic phosphate levels were not affected by either the level of vitamin D_3 or by use of autoclaved C-1 protein in the diet.

Increasing the vitamin D_3 level did not significantly alter tibia ash. However, there was a significant interaction ($P < 0.01$) between vitamin D_3 and autoclaving. The data suggest that the interaction was apparently due to the greater response of the poults fed autoclaved C-1 protein to the higher vitamin D_3 levels. Changes in vitamin D_3 levels for poults on untreated C-1 protein gave inconsistent results in tibia ash.

Serum alkaline phosphatase activity was significantly reduced ($P < 0.05$) by increasing the vitamin D_3 level from 880 to 1760 ICU/kg of diet. Increasing the vitamin D_3 level about 1760 ICU/kg of diet did not cause any further change in alkaline phosphatase activity. The data in table 2 also show that including autoclaved C-1 protein in the diet significantly lowered ($P < 0.05$) serum alkaline phosphatase activity. There was a significant interaction ($P < 0.05$) be-

TABLE 2

Influence of autoclaving (40 min) and vitamin D_3 levels on the rachitogenic property of C-1 protein

Vitamin D_3 level	Body wt gain			Tibia ash			Serum alkaline phosphatase activity			Serum inorganic phosphate			Serum calcium		
	Autoclaving [1] time			Autoclaving time			Autoclaving time			Autoclaving time			Autoclaving time		
	0 min	40 min	Mean [2]	0 min	40 min	Mean	0 min	40 min	Mean	0 min	40 min	Mean	0 min	40 min	
ICU/kg of diet	*g*	*g*	*g*	*%*	*%*	*%*	*Bodansky unit* [3]			*mg/100 ml*			*mg/100 ml*		
880	262 [4]	230	246	34.5	35.4	35.0	190	208	199[a]	5.6	6.0	5.8	10.2	10.0	10.1
1760	258	250	254	32.7	40.6	36.7	156	100	128[b]	4.9	5.6	5.3	9.9	9.3	9.6
3520	210	214	212	31.5	40.5	36.0	153	70	111[b]	5.5	6.5	6.0	10.6	9.5	10.1
7040	236	233	235	35.2	41.3	38.3	125	108	117[b]	6.4	7.8	7.1	9.7	10.2	10.0
Mean [5]	242	232		33.5	39.5*		156	122*		5.6	6.5		10.1	9.7	

[1] Autoclaved at 120°.
[2] Means in the same column bearing different superscript letters are significantly different at $P < 0.01$.
[3] One Bodansky unit = 1 mg inorganic phosphate released/100 ml serum/1 hr incubation at 37°.
[4] Mean of 3 pens/treatment.
[5] Means under each criterion bearing 1 asterisk are significantly different at $P < 0.05$.

TABLE 3

Influence of autoclaving (60 min) and vitamin D_3 levels on the rachitogenic property of C-1 protein

Vitamin D_3 level	Body wt gain			Tibia ash			Serum alkaline phosphatase activity			Serum inorganic phosphate			Serum calcium		
	Autoclaving [1] time			Autoclaving time			Autoclaving time			Autoclaving time			Autoclaving time		
	0 min	60 min	Mean [2]	0 min	60 min	Mean	0 min	60 min	Mean	0 min	60 min	Mean	0 min	60 min	Mean
ICU/kg of diet	*g*	*g*	*g*	*%*	*%*	*%*	*Bodansky unit* [3]			*mg/100 ml*			*mg/100 ml*		
880	149 [4]	354	252[a]	27.8	44.5	36.2[a]	237	73	155[a]	3.8	5.5	4.7	9.7	11.2	10.5
1760	257	401	329[b]	34.8	47.5	41.2[b]	157	39	98[b]	5.4	5.9	5.7	9.5	11.6	10.6
3520	328	433	381[c]	39.2	47.6	43.4[c]	101	62	81[c]	5.2	6.5	5.9	10.2	12.0	11.1
Mean [5,6]	254	396**		33.9	46.5*		165	58**		4.8	5.9		9.8	11.6*	

[1] Autoclaved at 120°.
[2] Means in the same column bearing different superscript letters are significantly different at $P < 0.05$.
[3] One Bodansky unit = 1 mg inorganic phosphate released/100 ml serum/1 hr incubation at 37°.
[4] Mean of 3 pens/treatment.
[5] Means under each criterion bearing 1 asterisk are significantly different at $P < 0.05$.
[6] Means under each criterion bearing 2 asterisks are significantly different at $P < 0.01$.

tween autoclaving and vitamin D_3 level. This was apparently due to the greater response to changes in vitamin D_3 level exhibited by the poults on autoclaved C-1 protein, especially at the lower level of vitamin D_3.

Experiment 2. This experiment was conducted to determine whether a longer period of autoclaving would be more effective than the 40-minute period used in experiment 1. The data for experiment 2 are presented in table 3.

As in the first experiment, serum inorganic phosphate was not significantly altered by autoclaving C-1 protein nor by increasing the vitamin D_3 level. In contrast with the first experiment, serum calcium was significantly increased ($P < 0.05$) as a result of autoclaving the C-1 protein, though increasing vitamin D_3 levels did not markedly alter serum calcium levels. Body weight gains were significantly increased by each increment of vitamin D_3 and by autoclaving, at $P < 0.05$ and $P < 0.01$ levels, respectively. Serum alkaline phosphatase activity was significantly decreased by each increment of vitamin D_3 and by autoclaving, at $P < 0.05$ and $P < 0.01$ levels, respectively. Tibia ash was also significantly increased ($P < 0.05$) by each increment of vitamin D_3 and by autoclaving the C-1 protein. Statistical analysis of the data revealed that for tibia ash and alkaline phosphatase activity there was a significant interaction ($P < 0.05$) between autoclaving and level of vitamin D_3. In both criteria little additional response was obtained from the highest level of vitamin D_3 (3520 ICU/kg of diet) with the autoclaved C-1 protein, whereas all vitamin additions produced considerable response in poults fed the untreated protein.

Experiment 3. The results from experiments 1 and 2 indicated that an autoclaving time of 60 minutes was more effective than one of 40 minutes. It was the purpose of this experiment to re-evaluate the effect of various periods of autoclaving within the same study. The data are shown in table 4.

As in experiments 1 and 2, serum inorganic phosphate was not affected by either autoclaving or increasing vitamin D_3 levels.

TABLE 4

Effect of varying autoclaving time and vitamin D_3 levels on the rachitogenic property of C-1 protein

Autoclaving[1] time	Body wt gain			Tibia ash			Serum alkaline phosphatase activity			Serum inorganic phosphate			Serum calcium		
	Vitamin D_3, ICU/kg			Vitamin D_3, ICU/kg			Vitamin D_3, ICU/kg			Vitamin D_3, ICU/kg			Vitamin D_3, ICU/kg		
	1760	3520	Mean[2]	1760	3520	Mean	1760	3520	Mean	1760	3520	Mean	1760	3520	Mean
min	*g*	*g*	*g*	*%*	*%*	*%*	*Bodansky unit*[3]			*mg/100 ml*			*mg/100 ml*		
0	181[4]	209	195[a]	29.6	30.7	30.2[a]	163	159	161[a]	7.2	5.0	6.0	8.0	9.1	8.6[a]
20	281	218	250	32.0	32.3	32.2	153	123	135	4.2	4.7	4.5	9.6	9.1	9.4
40	318	312	315[b]	31.1	35.0	33.1[b]	131	113	122	4.9	4.9	4.9	10.1	10.5	10.3[c]
60	309	412	361[b]	39.4	40.0	40.0[c]	96	99	98[c]	4.6	4.5	4.5	10.8	10.6	10.7[c]
Mean	272	287		33.0	35.0		136	124		5.2	4.8		9.6	9.8	

[1] Autoclaved at 120°.
[2] Means in the same column bearing superscript letters *b* and *c* are significantly different from the control, *a*, at $P < 0.05$ and $P < 0.01$, respectively.
[3] One Bodansky unit = 1 mg inorganic phosphate released/100 ml serum/1 hr incubation at 37°.
[4] Mean of 3 pens/treatment.

Increasing the vitamin D_3 level from 1760 to 3520 ICU/kg of diet did not affect any of the observations made. Although increasing vitamin D_3 levels did not affect the overall serum calcium means, there was a significant interaction ($P < 0.05$) between autoclaving and vitamin D_3. The poults on the lower vitamin level (1760 ICU/kg of diet) exhibited the greater response to autoclaving.

Although weight gains, tibia ash and serum calcium values appeared to be affected by feeding C-1 protein which had been autoclaved for 20 minutes, the first statistically significant response ($P < 0.05$) was produced by feeding C-1 protein which had been autoclaved for 40 minutes. Increasing the autoclaving time to 60 minutes increased the response in each of the criteria although the further changes were not statistically significant in all cases. There was a continuous decrease in alkaline phosphatase activity as the time of autoclaving the C-1 protein was increased; however, 60 minutes of autoclaving was required to give the first statistically significant ($P < 0.01$) response.

DISCUSSION

These investigations indicate that the rachitogenic activity of C-1 protein was markedly reduced by autoclaving under the conditions described. Sixty minutes of autoclaving gave the greatest destruction of the rachitogenic properties of the protein, although shorter treatment periods had some effect.

Feeding autoclaved C-1 protein did not markedly affect serum inorganic phosphate, whereas it caused varied responses in weight gains and serum calcium between different experiments. However, tibia ash values and serum alkaline phosphatase activity were affected similarly in each of the experiments. Inclusion of autoclaved protein in the diet always caused a significant increase in tibia ash and a concomitant decrease in serum alkaline phosphatase activity. Therefore, it appears that the level of serum alkaline phosphatase may in fact be related to the extent of rickets in poults, and the measurement of phosphatase activity may be useful in detecting the onset or alleviation of rickets within a group of poults. Since serum alkaline phosphatase levels are influenced by several other physiological disorders and little is known concerning the normal phosphatase levels in turkeys, it would be difficult to use this criterion as an absolute measure of rickets. From these 3 experiments, however, it appears that a serum alkaline phosphatase activity equal to 100 Bodansky units is equivalent to tibia ash values of 39% or greater.

The effect of feeding autoclaved C-1 protein on weight gains varied. In experiments 2 and 3 increased weight gains were obtained; however, in experiment 1 autoclaving the C-1 protein had no effect on weight gains. The reasons for this variability are not fully understood. However, differences in the initial nutritional status of the groups of poults may be a contributing factor to the variations observed between the experiments.

Serum calcium was increased in 2 out of 3 experiments by feeding autoclaved C-1 protein. This is somewhat unusual since serum calcium levels are quite resistant to change. The tibia ash values of poults fed untreated protein were altered markedly by increasing the vitamin D_3 levels without any effect being produced on serum calcium levels (table 3). Whether the increase in serum calcium brought about by autoclaving represented an increased absorption of calcium could not be ascertained from this study.

Under the conditions of these experiments serum inorganic phosphate levels appeared to be unrelated to the extent of rickets. They were not affected by either treatment.

The effects obtained by varying vitamin D_3 levels were similar in all 3 experiments although the changes were not statistically significant in experiment 3. However, in experiment 3, the vitamin D_3 levels were both high. Therefore, less response might be expected with the addition of more vitamin to an already high level. Increasing vitamin D_3 levels increased tibia ash values and decreased alkaline phosphatase activity. These responses were similar to those obtained by feeding autoclaved C-1 protein. Whether the mode of action of these 2 treatments is the same is

conjecture at this time. In any event, autoclaving C-1 protein for 60 minutes was more effective in reducing the rachitogenic properties of C-1 protein than the addition of vitamin D_3 (4 times NRC recommendations). It is conceivable that the vitamin D_3 merely exerts an overriding effect on the rachitogenic factor(s) in C-1 protein, whereas autoclaving involves the actual destruction of the factor(s). Although serum calcium levels were increased in 2 out of 3 experiments by feeding autoclaved C-1 protein, increasing vitamin D_3 levels did not affect serum calcium markedly. The possibility exists, therefore, that the effect of autoclaving is mediated by a pathway different from that involved in the control of rickets by vitamin D_3 additions.

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Effects of Dietary DL-Methionine on Tissue Levels of Glutathione in Hypothyroid Chicks[1]

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ABSTRACT Earlier studies have shown that dietary DL-methionine counteracts various effects associated with hyperthyroidism in chicks. A study has now been conducted to ascertain whether methionine counteracts, or augments, effects associated with hypothyroidism in chicks. The principal criteria were blood, liver, heart and muscle content of reduced glutathione (GSH). The hypothyroid state was induced by incorporating 6-propylthiouracil (PTU) into the diet. Its administration led to consistent increases in liver and breast muscle GSH, as did that of methionine. Combined supplementation led in nearly every case to still higher GSH levels. The data on heart, and particularly blood, are less clear-cut. On balance, though, they also support an interpretation of augmentation, rather than counteraction, of effects of methionine and PTU. Thus it has been shown that methionine synergizes, rather than antagonizes, the effects of hypothyroidism on tissue levels of GSH. This lends credence to the hypothesis of a specific antithyrotoxic effect of methionine.

Previous reports (1–4) have indicated dietary methionine to be an antithyrotoxic agent in chicks. This conclusion is based on the demonstration that methionine exerts effects in a direction consistently opposite from that of thyroactive materials (desiccated thyroid, iodinated casein), as judged by various criteria. Criteria used have included body weight gain, feed utilization efficiency, oxygen consumption in vivo, liver weight relative to body weight, and reduced glutathione (GSH) content of blood, liver, heart and muscle.

It occurred to us that an agent which ameliorates the effects of too much thyroid might be expected to aggravate the effects of too little. Hence we have measured the ability of methionine to do so, in chicks rendered hypothyroid by the use of the antithyroid drug, 6-propylthiouracil (PTU). This agent acts by inhibiting synthesis of thyroid hormones by the thyroid gland (5, 6), thereby producing in a fairly clear-cut manner a simple deficiency of thyroid hormone supply to the peripheral tissues.

MATERIALS AND METHODS

All experiments reported here were conducted in one-day-old Single Comb White Leghorn type of cockerel chicks from commercial hatcheries. The basal diet was MS-1 of this laboratory which is deficient in methionine-cystine, but otherwise nutritionally complete.

The percentage composition of this diet is as follows: yellow corn meal, 66; soybean meal (50% protein), 13.5; dehydrated alfalfa meal (17% protein), 5.88; dried brewer's yeast, 5.00; gelatin, 4.00; steamed bone meal, 1.70; limestone, 1.00; DL-phenylalanine, 0.214; DL-tryptophan, 0.041; iodized salt, 0.500; KCl, 0.200; and $MgSO_4$, 0.242. Vitamins and trace minerals were added per kg of diet, as follows: (in milligrams) $MnSO_4$, 50; $FeSO_4$, 20; $CuSO_4$, 2.0; $ZnCl_2$, 0.2; $CoCl_2$, 0.2; pyridoxine·HCl, 2.5; folic acid, 0.50; vitamin B_{12}, 0.025; and biotin, 0.10. Calculations based on reported compositions of the feedstuffs used indicated this diet contained 19.7% protein, provided all non-added vitamins and minerals in abundance, and all essential amino acids except methionine-cystine at or above levels recommended by the NRC (7) for a 20% protein diet.

The chicks as received were given yellow corn meal for 2 days during which those developing vent-pasting, and runts, were culled.

Experimental groups were selected thereafter by a systematic body weight-equalization procedure, and placed in individually heated, thermostatically controlled, wire mesh-bottom, all-metal pens. The batteries

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were housed in an air conditioned constant-temperature chick room at 25°. They were given feed and water ad libitum.

Experimental criteria were body weight gain, feed utilization efficiency (g total body weight gain/g total feed consumed × 100), heart and liver weights relative to body weight, and GSH content of heart, liver, blood and breast muscle. GSH was measured by the method of Kay and Murfitt (8) as modified here for routine application. Five experiments are being reported, involving various levels of PTU and DL-methionine, and various combinations of these as the dietary variables.

RESULTS

The body weight gains and organ weights of the chicks in response to methionine and PTU, alone and in combination, are summarized for all 5 experiments in table 1. Methionine supplementation consistently produced an increase in body weight gain, and an increase in efficiency of feed utilization (FUE) for body weight gain. PTU supplementation had, in terms of these criteria, opposite effects. Comparison of the "combination" (methionine + PTU) groups to the PTU groups shows clearly that added methionine had no ability to counteract the growth depression caused by PTU; and may even have aggravated it slightly in experiments 3 and 5. Methionine tended to reinstate the reduced feed utilization efficiency caused by PTU, but only partially.

PTU supplementation caused large increases in liver weight relative to body weight (LPBW). This was apparently owing to a gross, general deposition of fat. The livers of PTU-supplemented chicks were of visibly lighter color and larger, relative to the total carcass. Methionine augmented these increases in LPBW in four of the five experiments. Heart weight relative to body weight (HPBW) was decreased by PTU, and by methionine in all experiments, and in two there was augmentation of this effect by combined supplementation.

TABLE 1

Response of Single Comb White Leghorn cockerels to methionine and 6-propylthiouracil (PTU) at 4 weeks

Exp.	Treatment	Body wt gain	Cumulative FUE [1]	LPBW [2]	HPBW [2]
		g	%		
1	Basal	177 (12)	31.4 (12)	2.80 (12)	
	+ Met (0.25%)	234 (12)	46.0 (12)	2.88 (12)	
	+ PTU (0.05%)	105 (12)	32.7 (12)	5.99 (12)	
	Combination	124 (12)	34.8 (12)	5.72 (12)	
2	Basal	172 (16)	27.6 (10) [4]	2.55 (19)	0.565 (19)
	+ Met (0.4%)	216 (17)	32.1 (11) [4]	2.26 (20)	0.550 (20)
	+ PTU (0.1%)	104 (17)	21.3 (11) [4]	4.59 (20)	0.501 (20)
	Combination	116 (17)	23.1 (11) [4]	5.45 (20)	0.440 (20)
3	Basal	176 (20)	30.8 (20) [4]	2.39 (6)	0.553 (6)
	+ Met (0.4%)	241 (20)	35.5 (20) [4]	2.25 (6)	0.466 (6)
	+ PTU	156 (38) [5]	28.8 (38) [4]	3.53 (12)	0.509 (12)
	Combination	123 (37) [5]	34.0 (37) [4]	5.12 (12)	0.417 (12)
4	Basal	128 (21)	27.6 (21)	2.40 (6)	0.506 (6)
	+ Met (0.4%)	181 (21)	39.9 (21)	3.28 (6)	0.414 (6)
	+ PTU	150 (41) [5]	Lost	2.64 (12)	0.477 (11)
	Combination	130 (40) [5]	35.9 (40)	4.68 (12)	0.433 (12)
5	Basal	152 (19)	35.9 (19)	2.61 (12)	0.538 (12)
	+ Met (0.4%)	211 (20)	44.6 (20)	2.55 (12)	0.511 (12)
	+ PTU	110 (40) [5]	31.7 (40)	4.98 (24)	0.456 (24)
	Combination	106 (38) [5]	32.0 (38)	5.41 (24)	0.452 (24)

[1] FUE is feed utilization efficiency (g total body weight gain/g total feed consumed × 100).

[2] LPBW is liver % of body weight; HPBW is heart % of body weight. Each figure under these heads is a composite of all observations throughout the experiment, since temporal effects were negligible; hearts were not taken in the first experiment.

[3] Numbers in parentheses indicate number of birds/group.

[4] At 7 weeks in these experiments.

[5] In these experiments 2 levels of PTU (0.05% and 0.1%) were averaged; hence the doubled numbers of chicks in the PTU and combination treatments.

TABLE 2

Tissue content of reduced glutathione (GSH) in hypothyroid chicks as affected by methionine

Exp.	Treatment	No. of chicks	GSH content			
			Liver	Breast muscle	Heart	Blood
			mg GSH/100 g or ml			
1	Basal	12	66	8.1		51
	+ Met (0.25%)	12	125	15		59
	+ PTU [1] (0.05%)	12	111	32		47
	Combination	12	147	22		57
2	Basal	20	51	16	57	53
	+ Met (0.4%)	20	79	22	62	61
	+ PTU (0.1%)	20	88	31	63	58
	Combination	20	144	30	65	50
3	Basal	6	67	12	63	60
	+ Met (0.4%)	6	103	18	71	69
	+ PTU	12 [2]	81	22	62	59
	Combination	12 [2]	152	27	66	60
4	Basal	6	35	7.0	44	60
	+ Met (0.4%)	6	112	26	77	75
	+ PTU	12 [2]	55	14	58	62
	Combination	12 [2]	172	28	76	66
5	Basal	12	54	7.7	43	31
	+ Met (0.4%)	12	120	13	60	59
	+ PTU	24 [2]	86	17	41	26
	Combination	24 [2]	155	19	56	47

[1] PTU indicates 6-propylthiouracil.
[2] Double the number in the other 2 treatments because 2 levels of PTU (0.05% and 0.1%) were available here. They were pooled because of no apparent difference in response. Two lower levels (0.01% and 0.02%) were discarded from the data of experiments 1, 3, 4 and 5 as giving only partial responses.

TABLE 3

Summary of t tests for significance of mean difference in reduced glutathione (GSH) content of tissues

Effect	Liver	Blood	Breast muscle	Heart
	calculated values of t			
Met > basal	5.79 ***	3.54 **	4.75 ***	2.45 *
PTU [1] > basal	5.57 ***	ns	4.13 ***	1.07
Met + PTU > basal	9.96 ***	1.49	9.14 ***	6.71 ***
Met + PTU > PTU	5.18 ***	3.57 **	0.83	2.39 *

[1] PTU indicates 6-propylthiouracil.
* Significant at 90% probability; ** significant at 95% probability; and *** significant at 99% probability level.

The data for tissue content of GSH, which are perhaps more meaningful criteria, are shown in table 2. Here the picture is clearly the same with respect to liver GSH and breast muscle GSH. PTU and methionine each independently caused higher levels of GSH. In general the combination of PTU and methionine gave values higher than those from either methionine or PTU supplementation alone. This was without exception in liver. Methionine, singly, tended to promote higher liver levels than PTU, singly. PTU, singly, tended to promote higher muscle levels than methionine, singly. But in both of these tissues there was definite augmentation of effects of combined supplementation.

PTU effects on levels of GSH in heart tissue and in blood were inconsistent, whereas methionine supplementtaion resulted in consistent increases in both tissues.

In general the data indicate that methionine and PTU usually augment each others' effects on tissue levels of GSH in chicks. This is borne out in table 3, which

presents calculated t values associated with pertinent mean differences pooled for all 5 experiments.

DISCUSSION

The importance of the foregoing results, in context, is that in earlier work (2–4) methionine counteracted the effects of excessive peripheral levels of thyroid hormones. In the present work methionine augmented the effects of peripheral deprivation of thyroid hormones in the tissues where such effects were obtained. Accordingly it is clear that in metabolism, methionine and the thyroid hormones must be exerting opposite effects in some part of the cellular machinery. The position of methionine as an antithyrotoxic agent is strengthened.

The exact nature of the fundamental metabolic adjustments leading to these overtly opposing effects is not clear. The findings to date show, however, that excessive levels of thyroid hormones lower the efficiency of energy utilization for gain, and that dietary DL-methionine prevents or repairs this loss.

This focuses attention on the electron transport system and disruption or integrity of the associated phosphorylation. The findings support the established concept (9, 10) that thyroid hormones are uncouplers of oxidative phosphorylation, and suggest that some metabolite derived from methionine in cells has the ability to prevent or reverse this uncoupling. Further studies are under way to determine whether the antithyrotoxic effect of methionine can be explained in these terms.

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Availability of Vitamin B_6 Vitamers Fed Orally to Long-Evans Rats as Determined by Tissue Transaminase Activity and Vitamin B_6 Assay [1,2]

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ABSTRACT To study 1) the availability of the 3 vitamin B_6-vitamers when fed orally, and 2) the relationships between the vitamin B_6-vitamer content of rat tissues to glutamic-pyruvic and glutamic-oxaloacetic transaminase activity, 8 groups of weanling rats of the Long-Evans strain were fed a vitamin B_6-deficient diet for 14 days with the following modifications: group 1, no supplement; groups 2, 3, 4, 5 μg of pyridoxal, pyridoxol and pyridoxamine, respectively (administered daily as oral supplements); groups 5, 6, 7, 15 μg of pyridoxal, pyridoxol and pyridoxamine, respectively (administered daily as oral supplements); and group 8, 4 μg of pyridoxol/g of diet. Generally the total vitamin B_6 and the 3 vitamer levels of the tissues (liver, kidney, brain, muscle and heart) from rats receiving the 15-μg supplements were higher than those receiving the 5-μg vitamer supplements. This same effect was observed with glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase enzyme activity. However, no great differences were observed either in tissue vitamer content or enzyme activity due to the form of the vitamer fed. Thus the data indicated that, when fed orally, the 3 vitamers were equally available to the rat. The rat tissues were more severely depleted of pyridoxol than the other 2 vitamers. The extent of tissue depletion of pyridoxamine was similar to the depletion of total vitamin B_6 content. Glutamic-pyruvic transaminase was depleted to a greater extent than glutamic-oxaloacetic transaminase.

In studying the relationships between tissue vitamin B_6 content and tissue transaminase activity for rats, it appeared that pyridoxamine was not as effective a vitamer as pyridoxal or pyridoxol (1). The question was raised, however, concerning the stability of this form in the diet. Therefore, in the current study the 3 vitamers were fed orally, and at 2 levels. Data obtained on vitamin B_6 content and transaminase activity in the tissues suggest that all 3 vitamer forms were equally effective in the rat.

MATERIALS AND METHODS

1. Animals and methods. Eight groups of 6 male weanling rats [3] of the Long-Evans strain were fed a vitamin B_6-deficient diet for 14 days with the following modifications: group 1, no supplement; groups 2, 3, and 4, 5μg of pyridoxal, pyridoxol and pyridoxamine, respectively (administered daily as oral supplements); groups 5, 6, and 7, 15 μg pyridoxal, pyridoxol and pyridoxamine, respectively (administered daily as oral supplements); and group 8, 4 μg pyridoxol/g of diet. These oral supplements were set at suboptimal levels to show, if possible, a dose-response relationship for the vitamers. The groups of rats fed 4 μg of pyridoxol/g of diet in the second week of feeding consumed approximately 10 g or 40 μg of pyridoxol a day. This, too was below the optimum daily requirement of 100 μg of pyridoxol per day or 60 to 70 μg/10 g of diet for the albino rat as suggested by Beaton and Cheney (2). However, the daily requirement for the Long-Evans strain used in this experiment may differ from that of the albino strain. The basal diet contained: (in percent) glucose, 73; devitaminized casein, 18; corn oil, 4; Hegsted salt mixture (3),[4] 4; and cod liver oil, 1. The vitamins added were: (μg/100 g diet) thiamine•HCl, 400; riboflavin, 800; Ca pantothenate, 2500; niacin, 4000 and

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[2] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967.

[3] Purchased from Blue Spruce Farms, Inc., Altamont, New York.

[4] Purchased from Nutritional Biochemicals Corporation, Cleveland.

choline, 100,000. Food and water were available ad libitum. The animals were killed and the tissues were analyzed for transaminase activity and tissue vitamin B_6 content after 2 weeks of feeding.

2. *Vitamin B_6 assay.* The procedures for sample hydrolysis, chromatography and microbiologic assay were those previously reported (1, 4).

3. *Transaminase activity.* Glutamic-pyruvic and glutamic-oxaloacetic transaminases were analyzed according to procedures previously described (5, 6).

RESULTS AND DISCUSSION

1. Growth curves. The growth curves for the 8 groups of rats are shown in figure 1. The least weight gain was evidenced by the rats fed the deficient diet and by those receiving 5 μg of pyridoxamine per day. The rats fed 4 μg pyridoxol/g diet showed the greatest weight gain.

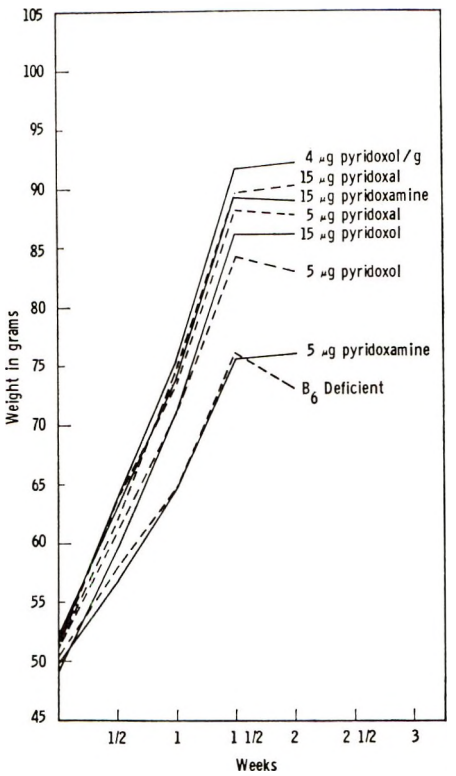

Fig. 1 Growth curves for body weights of rats maintained with a vitamin B_6-deficient diet with the following modifications: no supplement, 5 or 15 μg of pyridoxal, pyridoxol or pyridoxamine (administered daily as oral supplements) and 4 μg pyridoxol/g of diet.

2. *Tissue vitamer activity.* In tables 1, 2, and 3 are presented the data for the pyridoxol, pyridoxal and pyridoxamine activity, respectively, of liver, kidney, brain, muscle, and heart. It is shown in table 1, as previously reported (1, 4), that pyridoxol was the vitamer found in tissues in lowest concentration, although the supplemented groups had higher values than the deficient group. Vitamer supplementation and dietary treatment had little effect on the pyridoxol activity of these tissues. Pyridoxal content of the tissues also was not affected greatly by the various oral vitamer supplements as shown in table 2. However, the rats receiving 4 μg pyridoxol/g in the diet showed significantly higher values than the vitamin B_6-depleted group for liver, kidney and brain. Pyridoxamine activity of the rat tissues was affected the most by the various treatments as illustrated in table 3. Statistically higher values were observed in all tissues except heart with the groups receiving the 15-μg supplement for each vitamer and in those receiving the pyridoxol as a dietary constituent, when compared with the vitamin B_6-deprived group. Also, the kidneys of the rats receiving 5 μg of the vitamers showed significantly higher values than the vitamin B_6-deficient group.

The total vitamin B_6 activity of the tissues is shown in table 4. This represented the summation of the activities of the 3 vitamers. The highest levels of vitamin B_6 were found in liver and brain in the group receiving 4 μg pyridoxol/g of diet. With the other tissues one or more of the groups receiving the 15-μg oral vitamer supplements showed higher values than the group receiving pyridoxol in the diet. However, these differences were not very great. When comparing the tissue activities for the individual vitamers fed orally, either at the 5-μg or 15-μg level, no consistent differences were observed between them. This strongly suggested that they were equally available to the rat. Vitamer values at the 15-μg level of supplementation were generally higher than those at the 5-μg level, thereby demonstrating a dose-response relationship.

TABLE 1

Pyridoxol·HCl activity of rat tissues

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			μg/g tissue		
None	0.25 ± 0.12 [2]	0.13 ± 0.02	0.05 ± 0.02	0.12 ± 0.02	0.18 ± 0.04
5 μg pyridoxal [3]	0.37 ± 0.10	0.21 ± 0.04	0.11 ± 0.07	0.14 ± 0.04	0.36 ± 0.09
5 μg pyridoxol [3]	0.43 ± 0.16	0.23 ± 0.09	0.16 ± 0.05	0.14 ± 0.05	0.45 ± 0.12
5 μg pyridoxamine [3]	0.32 ± 0.13	0.22 ± 0.03*	0.09 ± 0.02	0.17 ± 0.04	0.49 ± 0.14
15 μg pyridoxal [3]	0.35 ± 0.09	0.33 ± 0.13	0.22 ± 0.15	0.35 ± 0.08*	0.58 ± 0.16*
15 μg pyridoxol [3]	0.36 ± 0.13	0.18 ± 0.31	0.12 ± 0.03	0.28 ± 0.08	0.38 ± 0.16
15 μg pyridoxamine [3]	0.33 ± 0.14	0.19 ± 0.04	0.09 ± 0.02	0.24 ± 0.08	0.41 ± 0.08*
4 μg pyridoxol /g diet	0.27 ± 0.10	0.23 ± 0.05	0.10 ± 0.03	0.17 ± 0.04	0.54 ± 0.15

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats and SE of mean.
[3] Vitamers were administered orally each day.
* Significantly different from the deficient mean, $P < 0.05$.

TABLE 2

Pyridoxal·HCl activity of rat tissues

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			μg/g tissue		
None	1.43 ± 0.07 [2]	0.62 ± 0.08	0.54 ± 0.06	1.74 ± 0.28	0.78 ± 0.08
5 μg pyridoxal [3]	2.32 ± 0.41	0.53 ± 0.09	0.51 ± 0.04	1.58 ± 0.23	0.46 ± 0.04**
5 μg pyridoxol [3]	2.13 ± 0.32	0.61 ± 0.07	0.52 ± 0.07	1.45 ± 0.20	0.69 ± 0.10
5 μg pyridoxamine [3]	1.85 ± 0.12*	0.61 ± 0.07	0.48 ± 0.04	1.58 ± 0.23	0.64 ± 0.05
15 μg pyridoxal [3]	2.44 ± 0.31**	0.73 ± 0.09	0.56 ± 0.08	2.08 ± 0.23	0.55 ± 0.07
15 μg pyridoxol [3]	2.16 ± 0.15**	0.71 ± 0.04	0.62 ± 0.07	2.67 ± 0.39	0.51 ± 0.05*
15 μg pyridoxamine [3]	2.10 ± 0.36	0.72 ± 0.08	0.59 ± 0.08	1.87 ± 0.32	0.49 ± 0.09*
4 μg pyridoxol/g diet	2.75 ± 0.25**	1.06 ± 0.08**	0.77 ± 0.07*	2.31 ± 0.28	0.64 ± 0.05

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats and SE of mean.
[3] Vitamers were administered orally each day.
** Significantly different from the deficient mean, $P < 0.01$.
* Significantly different from the deficient mean, $P < 0.05$.

TABLE 3

Pyridoxamine·HCl activity of rat tissues

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			μg/g tissue		
None	4.89 ± 0.30 [2]	1.96 ± 0.09	0.95 ± 0.10	0.83 ± 0.10	1.59 ± 0.19
5 μg pyridoxal [3]	6.23 ± 0.17**	3.26 ± 0.14**	1.16 ± 0.04	0.88 ± 0.11	1.62 ± 0.25
5 μg pyridoxol [3]	6.69 ± 0.97	3.28 ± 0.25**	1.17 ± 0.09	1.11 ± 0.12	1.29 ± 0.25
5 μg pyridoxamine [3]	4.92 ± 0.34	3.42 ± 0.11**	1.16 ± 0.05	0.87 ± 0.06	1.41 ± 0.23
15 μg pyridoxal [3]	6.43 ± 0.84	4.30 ± 0.20**	1.28 ± 0.07*	1.44 ± 0.03**	2.00 ± 0.25
15 μg pyridoxol [3]	6.84 ± 0.57*	4.56 ± 0.32**	1.39 ± 0.09**	1.46 ± 0.12**	2.27 ± 0.23
15 μg pyridoxamine [3]	7.46 ± 0.91*	4.53 ± 0.14**	1.35 ± 0.06**	1.49 ± 0.07**	2.26 ± 0.25
4 μg pyridoxol/g diet	6.90 ± 0.34**	4.32 ± 0.29**	1.48 ± 0.11**	1.38 ± 0.09**	1.93 ± 0.32

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats and SE of mean.
[3] Vitamers were administered orally each day.
** Significantly different from the deficient mean, $P < 0.01$.
* Significantly different from the deficient mean, $P < 0.05$.

3. *Tissue transaminase activity.* Glutamic-pyruvic transaminase activity and glutamic-oxaloacetic transaminase activity are given in tables 5 and 6, respectively. Glutamic-pyruvic transaminase was the enzyme most affected by dietary treatment as reported previously (1). Both in the liver and heart, glutamic-pyruvic transaminase values were higher for all the dietary modifications than for the deficient values. In muscle and kidney higher values were observed in the groups receiving 15 μg of the vitamers by oral supplementation. Dietary treatment resulted in no significant

TABLE 4

Total vitamin B_6 activity of rat tissues

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			μg/g tissue		
None	6.57 [2]	2.71	1.54	2.69	2.55
5 μg pyridoxal [3]	8.92	4.00	1.78	2.60	2.44
5 μg pyridoxol [3]	9.25	4.12	1.85	2.70	2.43
5 μg pyridoxamine [3]	7.09	4.25	1.73	2.62	2.54
15 μg pyridoxal [3]	9.22	5.86	2.06	3.87	3.13
15 μg pyridoxol [3]	9.36	5.45	2.13	4.41	3.16
15 μg pyridoxamine [3]	9.89	5.44	2.03	3.60	3.16
4 μg pyridoxol/g diet	9.92	5.61	2.35	3.86	3.11

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats.
[3] Vitamers were administered orally each day.

TABLE 5

Glutamic-pyruvic transaminase activity

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			mg pyruvic acid/g/hr		
None	35 ± 12 [2]	2 ± 0.33	5 ± 0.49	3 ± 0.86	4 ± 0.42
5 μg pyridoxal [3]	86 ± 13*	3 ± 0.58	7 ± 1.29	4 ± 1.2	7 ± 0.91**
5 μg pyridoxol [3]	81 ± 8**	2 ± 0.49	5 ± 1.44	5 ± 1.2	8 ± 1.09**
5 μg pyridoxamine [3]	70 ± 9*	3 ± 0.26	7 ± 1.09	6 ± 2.1	7 ± 0.34**
15 μg pyridoxal [3]	100 ± 17*	4 ± 0.40**	7 ± 0.56*	18 ± 4.6*	15 ± 1.14**
15 μ pyridoxol [3]	96 ± 11**	4 ± 0.36**	7 ± 1.18	10 ± 2.7*	14 ± 0.49**
15 μg pyridoxamine [3]	89 ± 20*	4 ± 0.43	7 ± 1.04	17 ± 3.5**	9 ± 0.48**
4 μg pyridoxol/g diet	71 ± 11*	4 ± 0.31**	6 ± 1.37	15 ± 4.4*	11 ± 1.16**

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats and SE of mean.
[3] Vitamers were administered orally each day.
** Significantly different from the deficient mean, $P < 0.01$.
* Significantly different from the deficient mean, $P < 0.05$.

TABLE 6

Glutamic-oxaloacetic transaminase activity

Diet supplement [1]	Liver	Kidney	Brain	Muscle	Heart
			mg pyruvic acid/g/hr		
None	158 ± 10 [2]	171 ± 16	476 ± 21	55 ± 17	63 ± 5
5 μg pyridoxal [3]	196 ± 18	169 ± 19	536 ± 43	123 ± 16*	48 ± 15
5 μg pyridoxol [3]	212 ± 14*	149 ± 11	440 ± 30	96 ± 6*	107 ± 53
5 μg pyridoxamine [3]	203 ± 12*	166 ± 10	536 ± 41	121 ± 15*	136 ± 43
15 μg pyridoxal [3]	223 ± 16**	192 ± 15	525 ± 32	152 ± 21**	108 ± 40
15 μ pyridoxol [3]	205 ± 16*	153 ± 18	560 ± 43	116 ± 14*	127 ± 20*
15 μg pyridoxamine [3]	221 ± 10**	180 ± 12	558 ± 46	160 ± 17**	73 ± 12
4 μg pyridoxol/g diet	204 ± 9**	244 ± 16**	453 ± 13	142 ± 12**	77 ± 16

[1] Vitamin B_6-deficient diet with the modifications indicated.
[2] Mean of 6 rats and SE of mean.
[3] Vitamers were fed orally each day.
** Significantly different from the deficient mean, $P < 0.01$.
* Significantly different from the deficient mean, $P < 0.05$.

changes in glutamic-pyruvic transaminase activity of the brain. The resistance of brain to change has been noted previously (1).

When glutamic-oxaloacetic transaminase was measured, higher values were observed in the liver and muscle with all the dietary modifications when compared with the vitamin B_6-deficient group. Oral supplementation of the vitamers resulted in no great differences in the glutamic-oxaloacetic transaminase activity of the kidneys, brains and hearts of these rats. Significantly higher enzyme activity was observed in the kidneys of rats receiving pyridoxol in the diet. There was no consistent difference in transaminase activity according to the

vitamer fed at each level, namely 5 μg or 15 μg. This suggested that the 3 forms were essentially equally available. However, enzyme activity was generally greater at the higher level of oral supplement, thereby indicating a dose-response relationship as found with tissue vitamer activity.

4. Relationship between tissue vitamin B_6 content and transaminase activity. In table 7 are presented calculated values for the extent of depletion of tissue vitamin B_6 content and transaminase activity for the rats receiving the 15-μg oral supplements. In most cases the percentage depletion was similar for each of the 3 vitamers fed. Thus the measurement of both tissue vitamin B_6 and transaminase activity indicated that pyridoxol, pyridoxal and pyridoxamine were equally available when administered as oral supplements. When the percentage depletion was calculated at the lower level, namely 5 μg of vitamer, the values were generally lower and more variable. With the ingestion per day of 40 μg of pyridoxol, assuming a daily consumption of 10 g of diet per rat, the greatest increase in percentage of depletion was noted in the tissue pyridoxal content.

It is also shown in table 7 that the tissues were more severely depleted of pyridoxol and the least depression was observed in tissue pyridoxal content. The extent of depletion of pyridoxamine closely paralleled that of total vitamin B_6 content. In all tissues glutamic-pyruvic transaminase enzyme activity was depressed in the vitamin B_6-depleted tissues to a greater extent than was glutamic-oxaloacetic transaminase enzyme. Glutamic-pyruvic transaminase has been found to be more sensitive to physiologic change by many others (7–9). The extent of depletion of glutamic-pyruvic

TABLE 7

Extent of depletion of various factors in rats fed 15 μg vitamer/day [1]

Factor assayed	Vitamer fed	Tissue					Horizontal avg
		Liver	Kidney	Brain	Muscle	Heart	
		%	%	%	%	%	%
Pyridoxol	pyridoxal	28.6	60.6	77.3	65.7	69.0	60.2
	pyridoxol	30.6	27.8	58.3	57.1	52.6	45.3
	pyridoxamine	24.2	31.6	44.4	50.0	56.1	41.3
	avg	27.8	40.0	60.0	57.6	59.2	48.9
Pyridoxal	pyridoxal	41.4	15.1	3.6	16.3	ne [2]	19.1
	pyridoxol	33.8	12.7	12.9	34.8	ne	23.5
	pyridoxamine	31.9	13.9	8.5	7.0	ne	15.4
	avg	35.7	13.9	8.3	19.4	ne	19.3
Pyridoxamine	pyridoxal	24.0	59.2	25.8	42.4	20.5	34.4
	pyridoxol	28.5	57.0	31.7	43.2	30.0	38.1
	pyridoxamine	34.4	56.7	29.6	44.3	29.6	38.9
	avg	29.0	57.6	29.0	43.3	26.7	37.1
Total vitamin B_6	pyridoxal	28.7	53.8	25.2	30.5	18.5	31.3
	pyridoxol	29.8	50.3	27.8	39.0	19.3	33.2
	pyridoxamine	33.6	50.2	24.1	25.3	19.3	30.5
	avg	30.7	51.4	25.7	31.6	19.0	31.7
Glutamic-pyruvic transaminase	pyridoxal	65.0	50.0	28.9	83.3	73.3	60.1
	pyridoxol	63.5	50.0	28.9	70.0	71.4	56.8
	pyridoxamine	60.7	50.0	28.9	82.4	55.6	55.5
	avg	63.1	50.0	28.9	78.6	66.8	57.5
Glutamic-oxaloacetic transaminase	pyridoxal	22.9	10.9	9.3	63.8	58.3	33.0
	pyridoxol	28.5	ne [2]	15.0	52.6	50.4	36.6
	pyridoxamine	22.5	5.0	14.7	65.6	13.7	24.3
	avg	24.6	8.0	13.0	60.7	40.8	31.3

[1] Calculated as:

$$\frac{\text{15 μg value} - \text{deficient value}}{\text{15 μg value}} \times 100.$$

[2] Indicates no effect.

transaminase activity was higher than the depletion of total vitamin B_6 content in all tissues except kidney. This effect was of the order of 2 to 3.5 times greater in liver, muscle and heart. However, the extent of depression of total vitamin B_6 content was higher than the depression of glutamic-oxaloacetic transaminase enzyme activity in liver, kidney and brain, the greatest difference being found in the kidney. The average extent of depletion of total vitamin B_6 content, though, was similar to that of glutamic-oxaloacetic transaminase enzyme activity.

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Incorporation of ^{75}Se-Selenomethionine and ^{35}S-Methionine into Chicken Egg White Proteins[1]

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ABSTRACT After the simultaneous injection of trace amounts of ^{75}Se-selenomethionine and ^{35}S-methionine into the wing vein of the hen, the extent and mode of incorporation of both amino acids into the egg white proteins was studied. The results obtained appear to indicate that the selenomethionine is incorporated in a manner indistinguishable from that of methionine. All of the ^{75}Se associated with the proteins was identified as ^{75}Se-selenomethionine. The amino acid appears to be bound to the protein by covalent linkages since it is not removed by dialysis, gel-filtration or precipitation by trichloroacetic acid. The ratio of ^{35}S/^{75}Se of the total egg white proteins is approximately maintained during several manipulations of the proteins including the isolation of crystalline ovalbumin and in the amino acids liberated during the proteolytic digestion of this protein. The only gross difference observed in the behavior of both amino acids is the absence from the protein hydrolysates of any ^{75}Se-selenocysteine although ^{35}S-cysteine was detected.

Nisman and Hirsch (1) have shown that a soluble fraction of *Escherichia coli* which contained aminoacyl-sRNA synthetases was capable of activating selenomethionine at an equal or possibly greater rate than that of methionine and that the selenomethionine was incorporated into protein. Hansson and Blau (2) have further demonstrated that ^{75}Se-methionine injected into the femoral vein of the cat appears in the proteins secreted by the pancreas. Olendorf and Kitano (3) investigating the fate of ^{75}Se-selenomethionine in humans found that much of it appears to be incorporated into the globulin fraction of the blood proteins.

In the above studies, no attempt was made to study simultaneously the incorporation of selenomethionine and methionine in order to assess whether their metabolic behavior and incorporation into protein was similar. Only in the report of Hansson and Blau was the nature of the compound containing ^{75}Se definitely shown to be intact selenomethionine.

The present study takes advantage of the large capacity of the laying hen to synthesize proteins during egg production (4) to study the comparative incorporation of trace ^{75}Se-selenomethionine and ^{35}S-methionine into egg white proteins. The nature of the compounds containing the radioactive isotopes was established by ion-exchange chromatography.

The labeled proteins obtained in these experiments serve as starting material for the studies on the digestion of exogenous and endogenous proteins in the gastrointestinal tract of the rat reported in a companion paper.

MATERIALS AND METHODS

^{75}Se-L-selenomethionine, specific activity 100 to 400 mCi/mmole and ^{35}S-L-methionine, specific activity 20 to 100 mCi/mg were obtained from the Radiochemical Centre, Amersham, Bucks. Chromatographic studies (see later) showed them to be more than 99% pure.

Incorporation of the labeled amino acids into the egg white proteins. Single Comb White Leghorn hens were housed in individual cages and fed ad libitum a commercial laying diet.[3] The radioactive amino acids dissolved in distilled water (0.04 to 0.2 ml) were injected into the wing vein immediately following the laying of an egg since it was desirable that the injection of

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3 Purina Laying Diet, Ralston Purina Company, St. Louis; supplemented with calcium carbonate.

the label coincide with the next ovulation (5).

Incorporation of the radioactive amino acids was studied in the egg layed on the day following the injection; in some cases, subsequent eggs, which were also labeled, were studied. The eggs were processed as soon as possible after laying by the following procedure. The egg white was separated from the yolk and filtered with suction through a sintered glass funnel to obtain a uniform suspension. This was cleared of membranes by centrifugation in a Servall centrifuge at 18,400 × *g* for 15 minutes and filtration with suction through filter paper of the supernatant. The filtrate was taken to a known volume with distilled water. In some studies crystalline egg albumin was obtained from this solution following essentially the procedure of Sorensen and Hoyrup (6). The ovalbumin thus obtained, dissolved in a minimum volume of water, was freed of any remaining ammonium sulfate either by dialysis against distilled water, or by passage through a Sephadex G-25 column (12 × 2 cm) by the technique describe below. The salt-free ovalbumin was then lyophilized and stored dry for further use.

Chromatography through Sephadex G-25 was performed in columns of 12 × 2 cm or 24 × 1 cm. The Sephadex G-25 was equilibrated with distilled water overnight and distilled water was used as the eluent. A flow rate of about 1 ml/minute was maintained by means of a Sigmamotor pump; 2-ml fractions were collected.

Identification of the form of the labeled amino acids present in the proteins. To identify the nature of the labeled amino acids present in the ovalbumin, attempts were made to protect any labeled cysteine and selenocysteine by converting them to the corresponding cysteic acids through their oxidation with performic acid. In view of the difficulties encountered with this technique, several different procedures were tested (7–10). In addition, the formation of carboxymethylcysteine and carboxymethylselenocysteine by the procedure of Anfinsen and Haber (11) was also studied. This latter procedure was modified in that the carboxymethylated protein was obtained from the reaction mixture by precipitation with trichloroacetic acid, conditions being the same as those used by Smith (10). Pure carboxymethyl-L-cysteine and carboxymethyl-DL-selenocysteine were prepared from L-cysteine and DL-selenocysteine [4] by reacting equivalent amounts of the amino acids with iodoacetate in a pH-stat,[5] maintaining the pH at 10 with 1 N NaOH and incubating at 25° until no further liberation of HI was observed.

The hydrolysis of the intact or modified ovalbumin was performed by the procedure of Moore and Stein (12). Amino acid analyses of the hydrolysates were performed in a Phoenix amino acid analyzer using the single column procedure of Piez and Morris (13). A preparative column was used and the effluent from the ion exchange column was split so that three-quarters of the effluent on its way to a fraction collector flowed through an anthracene cell (14) adapted to a Nuclear Chicago Liquid Scintillation Counter (model 720) fitted with a rate meter. The remaining one-quarter of the effluent was allowed to react with ninhydrin to obtain the concentrations of amino acids present. Simultaneous recording of the radioactivity and of the ninhydrin-color was obtained, although due to our arrangement, the ninhydrin peaks lagged behind the radioactive peaks by 15 minutes. (This difference was corrected in the figures shown).

Counting the labeled amino acids. The gamma emission of selenium was counted in a Nuclear Chicago well-counter fitted with a KI crystal. The radioactivity due to ^{35}S was counted in the Nuclear Chicago Liquid Scintillation counter (model 720) using 10 ml of the solution of Bray (15). When mixtures of ^{75}Se-^{35}S were present, however, the gamma emission of ^{75}Se was also counted in the above system. An equal sample of ^{75}Se-selenomethionine counted in the well-counter and in the liquid scintillation counter showed that 31% of the gamma counts were registered in the liquid scintillator. This relation allowed readily for subtraction of the ^{75}Se counts to be made when the mixed isotopes were

[4] Obtained from Sigma Chemical Company, St. Louis.

[5] Radiometer, Copenhagen.

counted. Quenching in the liquid scintillation counting was minor, corrections were made by adding the pure labeled amino acids as internal standards. Difficulties were encounted when attempts were made to determine the radioactivity of the labeled albumins in the above liquid scintillation system, since coagulation of the protein occurred. However, the labeled proteins could be counted either after hydrolysis, or in a Geiger-Muller counter,[6] after plating in copper planchets.

In this latter procedure, the gamma emission of ^{75}Se was nearly undetectable so that only a minor correction had to be made:

$$\frac{\text{count/min } ^{75}\text{Se in Geiger-Muller counter}}{\text{count/min } ^{75}\text{Se in the KI well-counter}} = 0.039.$$

RESULTS

Incorporation of labeled amino acids into the egg white proteins. In different experiments from 3.9×10^7 to 8.2×10^7 count/min of ^{75}Se-selenomethionine and 6.1×10^6 to 8.2×10^7 count/min of ^{35}S-methionine were injected into the hens. The recovery of the label in the egg white varied from 4.5 to 11.8%. In a typical experiment approximately 1.0 g of ovalbumin was obtained containing 1,520 count/min of ^{75}Se and 1,009 count/min of ^{35}S per mg of protein.

To establish the amount of the label in the total egg white actually bound to proteins, the following experiments were performed. A fraction of the egg white obtained from an egg laid before the injection of the labeled amino acids, was mixed with ^{35}S-methionine and ^{75}Se-selenomethionine and the mixture was applied to a Sephadex G-25 column (fig. 1 A.). All the ^{35}S and ^{75}Se radioactivity was obtained in the fraction retained by the gel and not with the excluded proteins. From the egg laid on the day following the injection into the same hen, of the labeled amino acids, a fraction of the egg white was applied to the column (fig. 1 B), all the ^{35}S and ^{75}Se appeared in the protein peak. Another fraction of the in vivo labeled egg white was exhaustively dialyzed against distilled water without any appreciable loss of label; neither did label appear in the supernatant of another radioactive egg white fraction

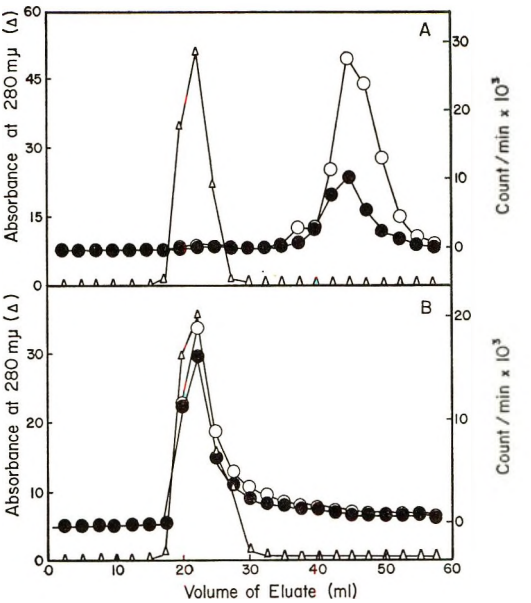

Fig. 1 Elution from a Sephadex G-25 column of egg white proteins labeled in vivo with ^{75}Se-selenomethionine and ^{35}S-methionine; *A*, 1 ml of an aqueous solution containing 100 mg of egg white proteins mixed in vitro with 102,197 count/min of ^{75}Se-selenomethionine and 36,790 count/min of ^{35}S-methionine, was introduced into the column; *B*, 1 ml of an aqueous solution of in vivo labeled egg white proteins containing 62 mg of protein and 80,910 and 59,064 count/min of ^{75}Se and ^{35}S, respectively, was introduced into the column. The original protein had a ^{35}S/^{75}Se ratio of 0.73; the 4 fractions of the eluate containing 91% of the applied radioactivity had ratios of 0.75, 0.69, 0.73 and 0.73.

precipitated with 10% trichloroacetic acid containing unlabeled methionine.

Nature of the radioactive compounds present in labeled ovalbumin. In agreement with McConnell and Wabnitz (16) it was observed that the elution of selenomethionine from the ion-exchange column of the amino acid analyzer is retarded when compared with that of methionine (fig. 2). It may be observed that ^{75}Se-selenomethionine is eluted just after the isoleucine peak. The greater basicity of the selenium also permits the differentiation between carboxymethylcysteine and carboxymethyl-Se-cysteine (fig. 3).

When attempts were made to protect cysteine or Se-cysteine present in the oval-

[6] Nuclear Chicago Model C-110-B; background was 4 counts/min.

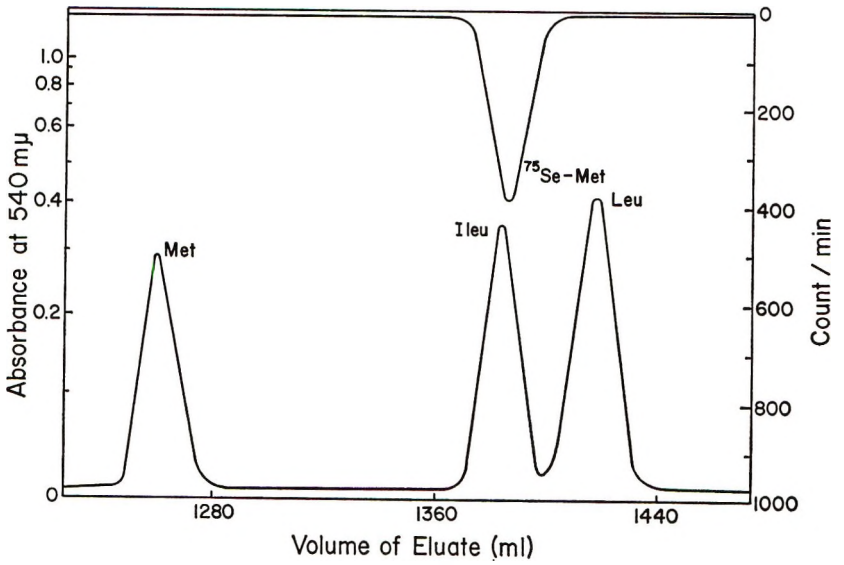

Fig. 2 Partial amino acid analysis of ovalbumin labeled in vivo with ^{75}Se-selenomethionine; 5 mg of ovalbumin containing 36,763 count/min of ^{75}Se were hydrolyzed (12) for 40 hours and applied to the amino acid analyzer. The radioactive peak of ^{75}Se-selenomethionine contained 35,734 count/min, a 97.2% recovery: for details see section on Methods.

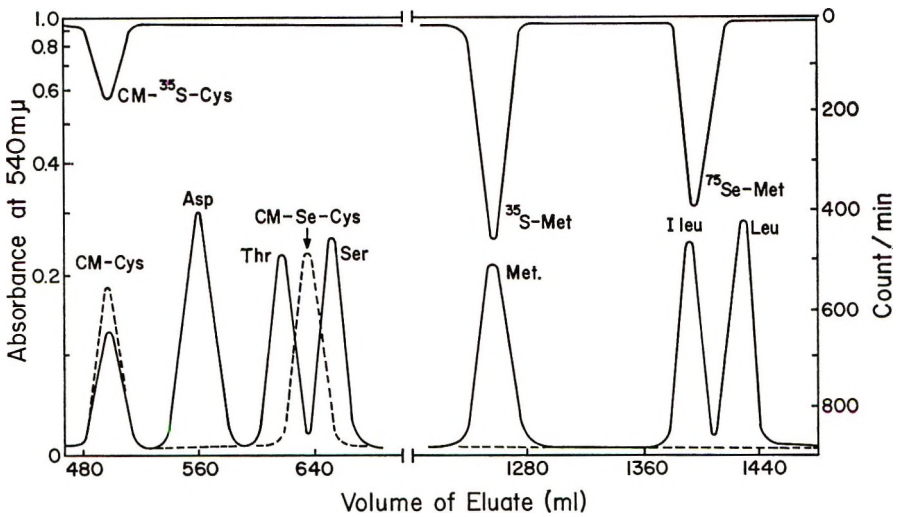

Fig. 3 Partial amino acid analysis of ovalbumin labeled in vivo with ^{75}Se-selenomethionine and ^{35}S-methionine; 20 mg of ovalbumin labeled with ^{75}Se and ^{35}S were treated after reduction, with iodoacetic acid to convert any cysteine and selenocysteine to the corresponding S-carboxymethyl (CM-Cys) or Se-carboxymethyl (CM-Se Cys) derivatives; 13.2 mg of the treated protein were hydrolyzed for 24 hours (12) and applied to the amino acid analyzer. ^{75}Se introduced 4,495 count/min recovered as ^{75}Se-selenomethionine 4,428 count/min (98.5%). ^{35}S introduced 1,385 count/min, 15% recovered in CM-Cys and 85% as methionine. The dotted lines indicate the position where pure CM-Cys and CM-Se-Cys are eluted: for details see section on Methods.

bumin by converting them to the corresponding cysteic acids, the oxidation of the protein with performic acid resulted in a marked destruction of the Se-labeled amino acids. Even though several techniques were tested (9–12), nearly 40% of the ^{75}Se was lost during the hydrolysis step (it appeared in the humin after filtration), and the remaining radioactivity was found in the elution through the amino acid analyzer in a number of peaks which could not be identified.

No loss of ^{75}Se was observed when the ovalbumin was hydrolyzed without any treatment or when the carboxymethyl derivatives were prepared by the method of Anfinsen and Haber (11). It is shown in figures 2 and 3 that nearly all (98.5%) of the radioactivity of ^{75}Se applied to the ion-exchange column appeared in the ^{75}Se-selenomethionine peak, while 85% of the ^{35}S appeared in the methionine peak and 15% in the carboxymethylcysteine peak (fig. 3).

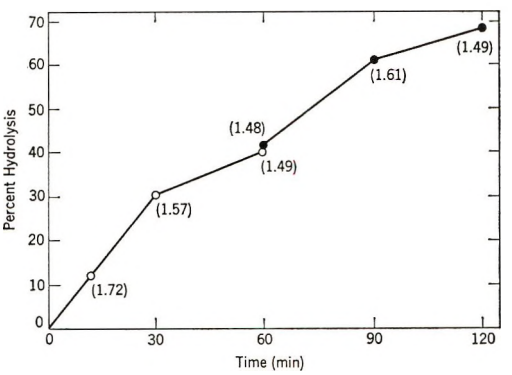

Fig. 4 Hydrolysis of ovalbumin labeled in vivo with ^{75}Se-selenomethionine and ^{35}S-methionine by pepsin and the combined action of trypsin, chymotrypsin and carboxypeptidase A; 200 mg of ovalbumin containing 101,388 and 165,262 count/min of ^{75}Se and ^{35}S, respectively, were dissolved in 2.0 ml of water containing 40 mg of pepsin, the solution was adjusted and maintained at pH 2.5 with 1 N HCl in a pH-stat during 60 minutes at 37°. At this time the pH was adjusted and maintained at 7.0 and 28 mg of trypsin, 28 mg of chymotrypsin and 14 mg of carboxypeptidase were added (arrow). At each experimental point, 0.2 ml of the incubation mixture was taken and the protein was precipitated by the addition of trichloroacetic acid to a final concentration of 10%. Numbers in parentheses indicate the ^{35}S/^{75}Se ratios in the liberated peptides and amino acids. Initial ratio was 1.63.

When the ovalbumin of the previous experiments was incubated with pepsin, followed by trypsin, chymotrypsin and carboxypeptidase A, (fig. 4), the ratio of the ^{35}S/^{75}Se in the supernatant after precipitation with trichloroacetic acid remained relatively constant. The first point recorded in the pepsin digestion gave low radioactivities in the supernatant due to the low degree of hydrolysis and this is thought to explain the somewhat higher value observed.

DISCUSSION

In one of the experiments performed, the ratio of ^{35}S/^{75}S in the radioactive amino acids injected into the hen was 0.61 and that observed in the labeled egg white proteins was of 0.73. This was a general finding, that is, a slightly greater incorporation of ^{35}S than of ^{75}Se into the proteins. This difference could be explained in part due to the fact that only ^{35}S-cysteine and no ^{75}Se-selenocysteine was found to be incorporated into the egg white proteins. The similarity in the observed ratios indicates that the fate of the selenomethionine is not grossly different from that of the natural amino acid.

The results obtained also appear to indicate that the selenomethionine is incorporated into the egg white proteins in a manner indistinguishable from that of methionine. The above can be concluded from the following evidence. a) All the ^{75}Se associated with the protein was identified as ^{75}Se-selenomethionine. b) The amino acid appears to be bound to the protein by covalent linkages since it is not removed by dialysis, gel-filtration or precipitation with trichloroacetic acid. c) The ratio of ^{35}S/^{75}Se or the total egg white proteins is maintained after passage of the proteins through a Sephadex G-25 column (fig. 1 B). In addition, the ratio is maintained in the crystalline ovalbumin obtained from the total egg white proteins. d) Whether the ^{75}Se-selenomethionine incorporated into the ovalbumin is present in the same positions in the peptide chains as those occupied by methionine can not be said with accuracy from the present findings. However, the fact that the peptides

and amino acids liberated by a series of proteolytic enzymes contain $^{35}S/^{75}Se$ ratios nearly equal to those of the original protein (fig. 4) indicates that if differences in location are present, they are not very great. Similar evidence can be obtained from the findings of parallel in vivo digestion of ^{75}Se-selenomethionine-^{35}S-methionine containing ovalbumin (17).

The only marked difference in behavior observed between the methionine and its Se-analogue is the absence of ^{75}Se-selenocysteine in the egg white proteins. In all the eggs studied the radioactivity in the proteins due to ^{75}Se was almost completely recovered as selenomethionine, and even though several procedures to protect any selenocysteine present were tried, no evidence of selenocysteine in the protein hydrolysates was obtained. This could be due to impaired conversion of ^{75}Se-selenomethionine into ^{75}Se-selenocysteine or, if the latter is formed, it does not appear to become incorporated into the proteins.

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Digestion and Absorption of Ingested and Secreted Proteins Labeled with ^{75}Se-Selenomethionine and ^{35}S-Methionine in the Gastrointestinal Tract of the Rat [1]

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ABSTRACT By labeling exogenous and endogenous proteins with different radioactive amino acids an attempt was made to further establish their fate in the gastrointestinal tract of the rat. Ovalbumin labeled with ^{75}Se-selenomethionine and ^{35}S-methionine was fed to rats and the rate of disappearance of both amino acids from the stomach and different segments of small intestine was studied. No differences could be observed in the rate of liberation during digestion and in the subsequent absorption of both amino acids. Similarly, a parallel rate of absorption of both methionines was observed from an intestinal segment in situ. Using ovalbumin labeled with ^{75}Se-selenomethionine, the extent of digestion of dietary versus endogenous secreted proteins showed that exogenous proteins emptied from the stomach are rapidly digested and that the small fraction not digested accumulates in the distal jejunum and ileum, together with the majority of the endogenous protein and of the tryptic activity present in the small intestine. In the presence of soybean trypsin inhibitor added to the diet, a marked reduction in the digestion of the exogenous protein resulted, while the endogenous protein content of the intestine remained relatively constant. Labeled endogenous proteins, peptides and amino acids were isolated from the stomach and the small intestine if radioactive methionine was injected intravenously 2 hours before feeding. This indicates that some digestion of endogenous protein was also taking place; however no quantitative estimates could be obtained.

The digestion of dietary proteins by mammals has been shown to require the secretion of large amounts of endogenous proteins (1–5). Nasset (1) has proposed that the dilution of the exogenous protein by endogenous proteins and their simultaneous digestion assures a constant amino acid pattern for the liver regardless of the nature of the ingested protein. An alternative explanation to Nasset's findings has been offered (6), namely that exogenous proteins are digested much more rapidly than endogenous protein and that due to the accumulation of the latter, a relatively constant amino acid pattern prevails in the contents of the gastrointestinal tract.

In the present study, by labeling exogenous and endogenous proteins with different radioactive amino acids an attempt was made to further establish their fate in the gut of the rat. Based on the findings presented in the previous paper, ovalbumin labeled with ^{75}Se-selenomethionine and with ^{35}S-methionine was used as the exogenous protein. To label the endogenous protein, advantage was taken of the findings of Hansson (7) and of Hansson and Blau (8) that intravenously injected radioactive amino acids, including ^{75}Se-selenomethionine, are rapidly incorporated into the protein of the pancreas, labeled proteins appearing in the pancreatic secretions within 1 to 2 hours after injection. In addition to the above, evidence is also presented indicating a parallel behavior of protein-bound ^{75}Se-selenomethionine and ^{35}S-methionine during in vivo digestion and absorption from the small intestine of the rat.

MATERIALS AND METHODS

Male rats of the Wistar strain weighing 200 ± 20 g were fed ad libitum a complete purified diet (9) for at least one month before their use. Food was removed 18 hours before experiment. After this period, they were offered for 5 minutes a complete diet (same as above) but, containing as the source of protein, ovalbumin labeled with the appropriate radioactive amino acid. The amount of diet consumed was recorded and the animals were left without food for

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a 30-minute period; the rats then received an injection of pentobarbital sodium [3] (4 mg/100 g of body weight) to prevent mucosal shedding (10) and 15 minutes later, the gastrointestinal tract was exposed and firmly ligated at the cardias, pylorus and at the end of the ileum. It was then removed from the animal and placed in an ice bath. The small intestine was immediately ligated and cut into 4 loops of about 21 cm each according to the procedure of Marcus and Lengemann (11). These segments represent, anatomically, the duodenum, jejunum, jejunum plus possibly some ileum, and ileum, respectively. The contents of the stomach and the intestinal loops were obtained by thoroughly flushing with distilled water; the collected contents and washings were taken to a total volume of 20.0 ml and were then homogenized in a Potter-Elvehjem glass pestle homogenizer. All operations were performed at 0–4°.

In the experiments designed to study the fate of the endogenous proteins, the labeled amino acid was injected into the tail vein of the rat 2 hours before offering food. Thereafter the procedure followed was the same as that described above.

The contents of the stomach and of the intestinal segments were analyzed further by the following procedures: To 14.0 ml of the contents of each segment of the gastrointestinal tract were added 1.5 ml of 75% aqueous trichloroacetic acid solution; after mixing and allowing to stand 15 minutes in an ice bath, the precipitated proteins were sedimented by centrifugation and the supernatant was removed; the residue was then washed twice each with 10% trichloroacetic acid solution, absolute ethanol, 1:1 ethanol-ether and ether. The proteins were then air-dried and finally dissolved in 1 N NaOH with heating,

The original contents and the dissolved proteins were assayed for total nitrogen by micro-Kjeldahl, and for radioactivity by the procedure described previously (12). The radioactivities in the material not precipitated by trichloroacetic acid were either counted directly or determined from the difference of the counts in the total contents and the precipitated proteins. Both procedures gave essentially the same results. In some experiments, the ^{75}Se counts were also determined in the intestinal wall by introducing the total organ or a fraction thereof into tubes and obtaining the count in a well-counter. The emission of ^{75}Se is energetic enough so that all the counts present were registered.

The intestinal contents of each segment were also assayed for trypsin activity by the method of Rick (13) using N-α-benzoyl-L-arginine ethyl ester as substrate.

The absorption of ^{75}Se-selenomethionine and ^{34}S-methionine from the intestine was studied essentially by the procedure of Gitler and Martinez-Rojas (14). The rats were anesthesized as indicated previously. The small intestine was exposed by a midline incision approximately 3 cm long; starting at a point 10 cm from the pylorus a sac 10 cm long was loosely ligated leaving the circulation intact. Through the distal ligature a fine polyvinyl capillary was introduced and the ligature was tightened. Through the proximal ligature was introduced 1 ml of a solution containing 2 μmoles of 18 L-amino acids (15) and trace ^{75}Se-selenomethionine and ^{35}S-methionine. The initial carrier methionine concentration was 2 mM. The loop was firmly ligated and returned to the peritoneal cavity leaving the open end of the capillary tube exposed. At the times studied 20-μliter samples were taken with a Hamilton syringe. The radioactivity of these was determined to establish the amount of the labeled amino acids absorbed.

The soybean trypsin inhibitor was a purified preparation.[4]

RESULTS

Figure 1 shows the in situ absorption from the rat small intestine of trace ^{75}Se-selenomethionine and ^{35}S-methionine present in an equimolar mixture of 18 amino acids. The results indicate a similar rate and extent of absorption of both amino acids.

When ovalbumin labeled with ^{75}Se-selenomethionine and ^{35}S-methionine is fed to a rat, the digestion and absorption of both labeled amino acids is similar (table 1); thus the $^{35}S/^{75}Se$ ratio of the ingested

[3] Nembutal, Abbott Laboratories, North Chicago, Illinois.

[4] Obtained from Sigma Chemical Company, St. Louis.

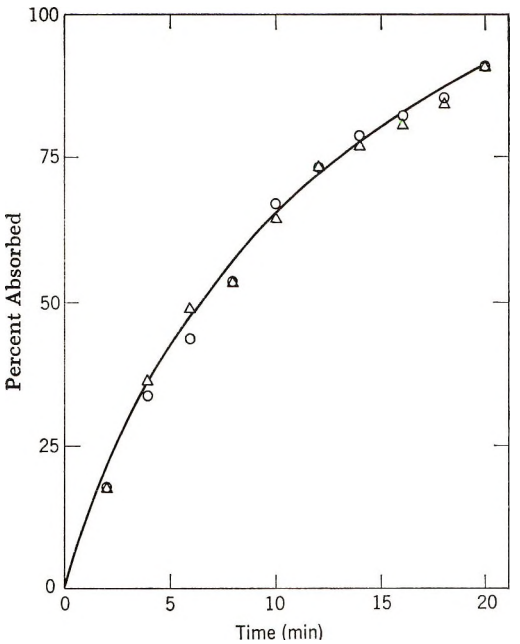

Fig. 1 The absorption from a loop of rat intestine in situ of trace ^{75}Se-selenomethionine (△) and ^{35}S-methionine (○) in the presence of a mixture of 18 L-amino acids (2 μmoles/ml of each). See Methods for experimental details; 1.18×10^6 count/min of ^{75}Se-selenomethionine and 4.37×10^5 count/min of ^{35}S-methionine were introduced into the loop.

protein is essentially equal to that found in the total and in the trichloroacetic acid-precipitable radioactivity of the stomach and of the 4 intestinal segments studied, even though some 80% of the labeled protein emptied into the intestine has been digested and the products absorbed. In this same experiment it can also be observed that the undigested protein tends to accumulate in the last 2 segments of the intestine, as does the tryptic activity. An appreciable quantity of endogenous protein was found throughout the tract, being greatest in the stomach and in the last 2 segments of the intestine, where it exceeded that of exogenous origin.

In the experiment shown in table 2, the injection of ^{35}S-methionine into the tail vein of the rat, 2 hours before feeding a diet containing ovalbumin labeled with ^{75}Se-selenomethionine, resulted in the appearance in the stomach and intestine of proteins, peptides and amino acids labeled with ^{35}S-methionine. This radioactive material is distributed throughout the tract, but was present in highest proportion in the stomach and in the last segment of the small intestine. The total endogenous protein in this experiment is more evenly distributed throughout the tract; however,

TABLE 1

Digestion and absorption of exogenous ovalbumin labeled with ^{35}S-methionine and ^{75}Se-selenomethionine [1]

Component studied	Intestinal segment				
	Stomach	1	2	3	4
Total protein (N × 6.25), mg	127	7.8	13.2	19.9	16.9
Total radioactivity due to ^{75}Se, count/min	155,040	3,464	5,856	11,728	12,752
Total radioactivity due to ^{35}S, count/min	35,496	785	1,492	2,703	3,032
Ratio ^{35}S/^{75}Se	0.23	0.23	0.25	0.23	0.24
Exogenous protein-equivalent, mg [2]	102	2.3	3.8	7.7	8.4
Endogenous protein-equivalent, mg [3]	25	5.5	9.4	12.2	8.5
TCA-precipitable ^{75}Se, count/min	99,225	1,916	4,361	9,509	10,296
TCA-precipitable ^{35}S, count/min	24,506	484	1,000	2,292	2,179
Ratio ^{35}S/^{75}Se	0.25	0.25	0.23	0.24	0.21
TCA-precipitable ^{75}Se, % of total radioactivity	64.0	55.3	74.5	81.1	80.7
TCA-soluble ^{75}Se, count/min	55,815	1,548	1,495	2,219	2,456
TCA-soluble ^{35}S, count/min	10,990	301	492	413	844
Exogenous protein-equivalent evacuated into the intestine, mg [4]	103	—	—	—	—
Exogenous protein-equivalent evacuated, not absorbed, % of total	—	2.2	3.7	7.5	8.2
Trypsin activity, % of total	—	1.1	1.1	47.9	49.8

[1] The rat ate 1.025 g of diet containing 205 mg of ovalbumin and 71,340 count/min of ^{35}S and 311,764 count/min of ^{75}Se. The specific activities of the ingested proteins were 348 and 1,520 count/min/mg of protein of ^{35}S and ^{75}Se, respectively, giving a ^{35}S/^{75}Se ratio of 0.23.

[2] Total radioactivity of ^{75}Se/(count/min of ^{75}Se/mg of diet protein).

[3] Total protein minus exogenous protein-equivalent.

[4] Ingested protein minus exogenous protein-equivalent found in the stomach.

TABLE 2

Digestion and absorption of exogenous and endogenous proteins labeled with ^{75}Se-selenomethionine and ^{35}S-methionine, respectively [1]

Component studied	Intestinal segment				
	Stomach	1	2	3	4
Total protein (N × 6.25), mg	77.3	13.9	8.9	18.1	25.9
Total radioactivity due to ^{75}Se, count/min	257,450	9,000	2,390	49,140	59,453
Exogenous protein-equivalent, mg [2]	53.2	1.9	0.5	10.2	12.3
Endogenous protein-equivalent, mg [3]	24.1	12.0	8.4	7.9	13.6
Total radioactivity due to ^{35}S, count/min	9,640	2,849	1,747	4,684	8,482
Exogenous protein-equivalent evacuated into the intestine, mg [4]	142.8	—	—	—	—
Exogenous protein-equivalent evacuated, not absorbed, % of total	—	1.3	0.4	7.1	8.6
TCA-precipitable ^{75}Se, count/min	186,022	4,166	1,157	17,728	23,700
TCA-soluble ^{75}Se, count/min	71,428	4,834	1,233	31,412	34,750
TCA-precipitable ^{75}Se, % of total radioactivity	72.2	46.3	48.4	36.1	41.5
TCA-precipitable ^{35}S, count/min	6,758	1,282	311	4,176	7,096
TCA-soluble ^{35}S, count/min	2,882	1,567	1,436	508	1,386
TCA-precipitable ^{35}S, % of total radioactivity	70.1	45.0	70.6	89.2	83.7
Trypsin activity, % of total	—	10.6	2.0	21.9	64.5

[1] The rat ate 0.98 g of diet containing 196 mg of ovalbumin and 9.47 × 10^5 count/min ^{75}Se (specific activity of 4,834 count/min/mg of protein). Two hours before feeding, the rat was injected 2.7 × 10^6 count/min of ^{35}S-methionine into the tail vein.
[2] Total radioactivity of ^{75}Se/(count/min of ^{75}Se/mg of diet protein).
[3] Total protein minus exogenous protein-equivalent.
[4] Ingested protein minus exogenous protein-equivalent found in stomach.

TABLE 3

Digestion and absorption of exogenous and endogenous proteins labeled with ^{75}Se-selenomethionine and ^{35}S-methionine, respectively, in the presence of soybean trypsin-inhibitor [1]

Component studied	Intestinal segment				
	Stomach	1	2	3	4
Total protein (N × 6.25), mg	267	8.4	13.2	27.3	59.4
Total radioactivity due to ^{75}Se, count/min	729,830	6,640	21,490	54,140	142,050
Exogenous protein-equivalent, mg [2,3]	203	1.8	5.9	15.0	39.5
Total radioactivity due to ^{35}S, count/min	38,497	1,422	2,282	4,169	8,901
Endogenous protein-equivalent, mg [4,5]	64	6.6	7.3	12.3	19.9
Exogenous protein evacuated to intestine, mg	122	—	—	—	—
Exogenous protein-equivalent evacuated, not absorbed, % of total	—	1.5	4.8	12.3	32.3
TCA-precipitable ^{75}Se, count/min	599,050	4,356	13,194	41,936	79,726
TCA-soluble ^{75}Se, count/min	130,780	2,284	8,296	12,204	62,324
TCA-precipitable ^{75}Se, % of total radioactivity	82.1	65.6	61.4	77.4	56.1
TCA-precipitable ^{35}S, count/min	15,208	668	1018	3397	7,540
TCA-soluble ^{35}S, count/min	23,287	754	1264	772	1361
TCA-precipitable ^{35}S, % of total radioactivity	39.5	47.0	44.6	81.5	84.7
Trypsin activity, % of total	—	2.9	6.1	10.8	80.2

[1] The rat ate 1.62 g of diet containing 325 mg of ovalbumin and 1.17 × 10^6 count/min of ^{75}Se-selenomethionine (specific activity 3,579 count/min/mg of protein) and 32.5 mg of soybean trypsin-inhibitor. Two hours before feeding, the rat was injected 2.7 × 10^6 count/min of ^{35}S-methionine into the tail vein.
[2] Total radioactivity of ^{75}Se/(count/min of ^{75}Se/mg of diet protein).
[3] Does not include the contribution due to the soybean trypsin-inhibitor.
[4] Total protein minus exogenous protein-equivalent.
[5] Includes the soybean trypsin-inhibitor.

it is still highest in the stomach and in the last segment of the intestine; the tryptic activity is found mainly in the last 2 segments of the intestine. In general the activity of trypsin parallels more closely the radioactivity due to ^{35}S than does the total endogenous protein. With respect to the exogenous protein, its behavior in this experiment closely resembles that of the experiment shown in table 1. Of 142.8 mg of exogenous protein emptied from the stomach into the intestine, some 85% was digested and absorbed; the material not digested accumulated in the last 2 segments of intestine.

A similar experiment was next performed (table 3), but the diet now contained, in addition, soybean trypsin inhibitor. A much larger proportion (51% in this experiment, 49.9 and 53.0 in 2 others) of the exogenous protein emptied into the intestine remained in the tract, especially in the last 2 segments of the intestine. The pattern of the endogenous protein was essentially the same as that of the previous experiment, with the exception of a larger amount found in the stomach. This, however, could be due to the higher ingestion of diet which occurred in the present experiment. An appreciable tryptic activity was observed throughout the tract, being in one experiment equal in magnitude and in 2 others some 60% of that found in the animals fed a diet without the soybean trypsin inhibitor.

When ^{75}Se-selenomethionine-labeled ovalbumin was fed, the radioactivity in the wall of the intestinal segments could readily be detected. In a typical experiment, segments 1 through 4 contained 4535, 10721, 21479 and 11029 counts/min, respectively. If the ^{75}Se-selenomethionine was injected into the tail vein of the rat, these same segments showed 16855, 13710, 12727 and 9528 counts/min, respectively. While the absolute amounts found varied in each experiment, the pattern of distribution was in all experiments essentially as shown.

In table 4 are summarized the findings of different experiments with respect to the content of exogenous and endogenous protein and of tryptic activity.

DISCUSSION

Spencer and Blau (14) have shown that the transport rates of ^{75}Se-selenomethionine and ^{35}S-methionine are identical across

TABLE 4

Distribution of exogenous and endogenous proteins and of trypsin in the contents of the gostrointestinal tract of the rat

Component	Protein content [1]		Tryptic activity [2]
	Exogenous	Endogenous	
	mg	*mg*	*units*
Stomach	87.6 (53.2–102.0) [3]	21.0 (15.0–25.0)	—
Intestinal segment:			
1	1.8 (1.4–2.3)	6.7 (4.4–12.0)	994 (120–2800)
2	2.7 (0.5–4.7)	7.1 (4.4–9.4)	630 (40–2400)
3	6.0 (1.0–10.2)	11.9 (7.9–15.2)	3025 (320–10,400)
4	6.6 (2.3–12.3)	11.4 (8.5–13.8)	5026 (1640–10,800)
Total in intestine	16.8 (7.4–23.9)	37.1 (25.2–50.4)	9675 (2120–26400)
% of exogenous protein emptied from stomach found in intestine	15.2 (11.2–21.5)	—	—

[1] The values were calculated on the basis of the specific radioactivity of the ingested protein; 5 experiments.

[2] A unit is the amount of enzyme which gives a change in OD of 0.001/min using the method of Rick (13); 13 experiments.

[3] Numbers in parentheses represent range.

everted hamster intestinal sacs. The present findings confirm their observations using an in situ intestinal preparation which has been shown to resemble more closely normal physiological conditions (14). In addition, no difference could be detected in the extent of digestion and absorption of the digestion products when ovalbumin labeled with both amino acids was fed to rats (table 1).

Marcus and Lengemann (11), in studying the rate of food propulsion through the intestine, observed a relatively rapid rate of transit in duodenum and upper jejunum (segments 1 and 2, respectively), while a marked reduction in the rate of passage occurs in the lower jejunum and ileum (segments 3 and 4, respectively). The absorption of the greatest portion of the dietary nitrogen (80–90%) has been reported (17, 18) to take place in the distal duodenum and upper jejunum. It would follow, therefore, that any protein emptied from the stomach which is not readily digested should accumulate in the lower jejunum and ileum (6). Similarly, pancreatic enzymes, which constitute the major portion of the intestinal endogenous protein, should be found in greatest proportions in the lower part of the tract if resistant to digestion.

The results presented show that the majority of the exogenous protein emptied from the stomach is readily digested and its products absorbed. The small fraction remaining in the tract is found mainly in the last 2 segments of the intestine, together with the majority of the endogenous protein and the tryptic activity (table 4). In all the experiments, an appreciable quantity of endogenous protein was found throughout the tract. In the intestine it exceeded that of exogenous origin, but this is clearly the result of the rapid rate of digestion of the exogenous protein. It is difficult to establish whether the endogenous protein was being digested simultaneously. The addition of soybean trypsin inhibitor (table 3) increased significantly only the exogenous protein content of the tract, the small increase in endogenous protein being accounted for possibly by the contribution of soybean trypsin-inhibitor. In these experiments it is especially apparent that the decreased rate of exogenous protein digestion results in its accumulation (64%) in the ileum. Lyman et al. (19) have observed a stimulation of pancreatic secretion when diets containing soybean trypsin inhibitor are fed. This was not apparent in the present experiments. However, their experiments contained a crude inhibitor preparation at much higher levels than those used here, and Saxena et al. (20) have shown that separate soybean protein fractions are responsible for pancreatic hypertrophy and for trypsin inhibition.

Trichloroacetic acid-soluble material of endogenous origin was found in the stomach and in the intestinal segments (table 2). This could indicate that some digestion of these proteins is taking place (see also 5, 21), although it has been reported that nitrogenous compounds other than proteins are secreted into the gastrointestinal tract (22). It is known that endogenous proteins are digested since the excretion of protein in the feces is small (4). While the present results are suggestive that the digestion of endogenous protein is slower than that of exogenous origin, and that this digestion occurs mainly in the ileum, no definite proof can be given at present. However, support for this view can be obtained from the results of Goldman and Guggenheim (23) who studied the pattern of amino acids appearing in the intestinal lumen and in the portal plasma after feeding various proteins to rats. These authors found that at short-term intervals (30 minutes) following the meal, the concentration of free amino acids in the lumen and in portal plasma correlated with the amino acid content of the ingested proteins, indicating that they were not markedly modified by the contribution from the digestion of the endogenous proteins.

These results emphasize the usefulness of ^{75}Se-selenomethionine for the study of the metabolic fate of labeled proteins.

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Evidence of Malfunctioning Blood-Brain Barrier in Experimental Thiamine Deficiency in Rats [1,2]

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ABSTRACT Labeling patterns in the free glutamic acid from brain tissue from rats injected with sodium pyruvate-2-^{14}C indicate that the blood brain barrier is not functioning normally in thiamine deficiency. This alteration in selectivity permits pyruvic acid to enter the brain. The polyneuritis in deficiency, therefore, might result from altered metabolic pathways in the brain.

The prolonged lack of sufficient thiamine has been shown to cause polyneuritic, cardiovascular, gastrointestinal, and muscular disorders, yet the biochemical mechanisms underlying these abnormalities are still unknown. Ever since the classic work of Peters (1) using pigeons, thiamine deficiency has been described as a well-defined "biochemical lesion." This deficiency of thiamine apparently diminishes the organism's ability to decarboxylate pyruvic acid. This impairment in pyruvate oxidation and the resulting loss of energy is thought by many to account for the physiological changes, particularly the polyneuritis, observed in thiamine deficiency.

Numerous investigators (2–6) have shown that pyruvate oxidation is not diminished in thiamine deficiency in animals other than pigeons; thus some other mechanism must be responsible for the manifestations of thiamine deficiency. Labeling patterns in brain glutamic acid in vivo after administration of radioactive pyruvate or its precursors have been used to show the routes of pyruvic acid metabolism (7). Pyruvate is metabolized in mammalian tissue via acetyl CoA, the precursor of carbons 4 and 5 of α-ketoglutarate or via a dicarboxylic acid, the precursor of carbons 1, 2 and 3 of α-ketoglutarate. This α-ketoglutarate can then be converted to glutamate with no mixing of the labeled carbons. When sodium pyruvate-2-^{14}C is used, the resulting acetyl CoA will be carboxyl-labeled, which will label glutamate only in carbon 5 on the first turn of the Krebs cycle. Subsequent turning of the cycle labels only carbon 5 and 1. Conversion of pyruvate to oxaloacetate and thence to glutamate will label carbons 2 and 3 of glutamate with carbons 1, 2 and 3 being labeled on further cycling. Labeling in carbon 4 can only come from conversion of the original pyruvate to oxaloacetate or malate and thence to a precursor of pyruvate as in gluconeogenesis. On conversion of such an intermediate to pyruvate, carbons 2 and 3 will be labeled, which will result in acetyl CoA-1,2-^{14}C. Conversion of this doubly labeled acetyl CoA will label carbons 4 and 5 of the glutamate. In the present study these labeling patterns were used to study the pyruvate metabolism of normal and thiamine-deficient rats. The results suggest that the blood-brain barrier may be malfunctioning or degenerating in thiamine deficiency which could be responsible for the polyneuritis.

EXPERIMENTAL

Growth and maintenance of animals. Male rats of the Sprague-Dawley strain, with initial weights of 50 to 65 g, were fed a thiamine-deficient diet as described by Pearson et al. (8). As controls, animals pair-fed a thiamine-adequate diet were used. The deficient animals showed the characteristic deficiency signs: loss of appetite, loss of weight, weakness, development of a curvature of the spine, and they dragged their hind quarters. No polyneuritic symptoms were observed in the pair-fed control animals. When the thiamine antimetabolite, oxythiamine, was used, it

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was incorporated in the diet in a 50:1 ratio to the thiamine. These animals did not develop the polyneuritis observed in the thiamine-deficient animals; however they did develop the remaining deficiency characteristics.

When the first signs of polyneuritis occurred, the animals along with their pair-fed controls were injected via the femoral vein with 0.2 ml of a solution containing 0.2 mg (10 μCi) sodium pyruvate-2-^{14}C. Ten minutes after receiving the isotope, the animals were decapitated. The brains were removed rapidly, usually within one minute, blotted on filter paper, weighed and frozen in liquid nitrogen.

Isolation of brain glutamic acid. The brain tissue from each animal was homogenized with 1.3 ml of 0.6 N $HClO_4$ per g of tissue (9). The precipitate was removed by centrifugation and rehomogenized with a volume of 0.33 N $HClO_4$ equal to that of the first supernatant solution. The supernatant solutions were combined, neutralized with 2 N KOH, refrigerated overnight, and centrifuged to remove $KClO_4$. The neutral perchlorate-free filtrates were passed over a Dowex-1-acetate column, washed with water, and eluted with 0.5 N acetic acid (10). This method readily separates aspartic and glutamic acids from the other acid components of brain. The glutamic acid fraction was removed, diluted to 50 ml with water, and quantitatively determined using the ninhydrin method of Rosen (11). To 40 ml of this glutamic acid solution were added 2 mmoles (294 mg) unlabeled glutamic acid and evaporated to dryness at 60° under an air jet.

Degradation of glutamic acid. The glutamic acid was degraded by the Schmidt reaction as described by Mosback et al. (12), as modified by Hill et al. (13). Chloramine T was used to oxidize the glutamic acid to succinic semialdehyde, which was then reduced with hydrazine to butyric acid. The butyric acid was isolated and purified by steam distillation and Celite chromatography. Decarboxylation of the butyric acid was carried out using hydrozoic acid, resulting in carbon dioxide, representing carbon 5 of the glutamic acid, and propylamine. The amine was oxidized with alkaline permanganate to propionic acid. The propionic acid was steam distilled and purified by Celite chromatography. By alternate application of the Schmidt reaction to produce carbon dioxide and the next lower amine, which was oxidized to the corresponding acid, methylamine was finally obtained. The methylamine was distilled into hydrochloric acid. The hydrochloride was obtained by evaporation to dryness and then combusted to yield carbon 2 of glutamic acid as carbon dioxide. In each step the liberated carbon dioxide was collected, measured manometrically and the radioactivity determined in a vibrating reed electrometer.

Rates of gluconeogenesis. Perfusion studies were carried out using the apparatus and procedures as described by Exton and Park (14). The perfusate contained 30 g of bovine serum albumin per liter of Krebs-Henseleit bicarbonate buffer and sufficient washed red blood cells from normal rats to give a hematocrit of 22%. Sodium pyruvate-2-^{14}C was infused into the perfusate at a rate necessary to maintain a pyruvate concentration of 10 mM at constant specific activity. The rate of incorporation of radioactivity into glucose was considered a reflection of the rate of gluconeogenesis.

RESULTS AND DISCUSSION

The radioactive labeling in carbon 4 of the free glutamic acid in brain tissue can be useful in determining whether a given

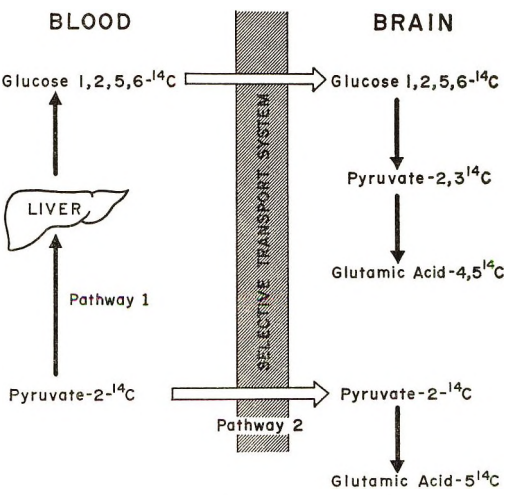

Fig. 1 Pathway 1 indicates entry through gluconeogenesis; pathway 2 indicates direct entry.

compound entered the tissue directly or by way of liver gluconeogenesis before entry (fig. 1). If sodium pyruvate-2-^{14}C was converted to glucose via the liver (pathway 1), the glucose would be labeled in the 1, 2, 5, 6 positions. This heavily randomized glucose would now result in abundant label in carbon 4 of the brain glutamic acid. If it entered the brain directly across the blood brain barrier (pathway 2) the glutamic acid would be labeled mainly in carbon 5. This technique was used by Koeppe and Hahn (15) to show that pyruvate does not enter the brain of adult animals. These results were confirmed by McMillan and Mortensen (16) in their labeling pattern studies on intracisternal injections of pyruvate-2-^{14}C. The results in table 1 show that pyruvate-2-^{14}C entered the brain directly in adult thiamine-deficient animals. The percentage randomization from pyruvate-2-^{14}C in thiamine-deficient rats is comparable to that obtained when glucose-2-^{14}C, a precursor of pyruvate-2-^{14}C, is used in normal rats. Thus, it is apparent that the selective transport system was not functioning in a normal fashion.

Since animals treated with oxythiamine do not develop the polyneuritis, it was of interest to investigate whether pyruvic acid entered the brain directly in these animals. It is shown in table 2, that the percentage randomization is comparable to that for the pair-fed controls. Oxythiamine apparently does not penetrate the blood brain barrier whereas thiamine does (18). When fed in combination with thiamine, there is a natural separation of the 2 compounds. The thiamine entering the brain is then sufficient to maintain brain metabolism and brain function.

It might be argued that the radioactivity in carbon 4 of the brain glutamic acid could also be affected if rates of gluconeogenesis were different in deficient and normal animals. If the rates of gluconeogenesis were slower in the deficient animal, the circulating pyruvic acid-2-^{14}C might have greater opportunity to penetrate directly by exchange or diffusion. To investigate this possibility, liver perfusion

TABLE 1

Distribution of ^{14}C in carbons 4 and 5 of brain glutamic acid after injection of pyruvate-2-^{14}C and glucose-2-^{14}C

Compound injected	Nutritional state	Total radioactivity in glutamate	Avg % of total radioactivity in carbon atoms		Randomization in carbon 4 [1]
			no. 4	no. 5	
		μCi/mmole			%
Pyruvate-2-^{14}C	normal diet ad libitum (10) [2]	1.52	21	56	55
Pyruvate-2-^{14}C	thiamine-deficient diet ad libitum (15)	2.45	2.4	56	8.2
Pyruvate-2-^{14}C	normal diet pair-fed (15)	2.4	8.7	46	32
Glucose-2-^{14}C	normal diet	3.0	3.1	57	10

[1] Calculated according to the method of Shreeve (17).
[2] Numbers in parentheses indicate number of animals used.

TABLE 2

Distribution of ^{14}C in carbons 4 and 5 of brain glutamic acid after administration of pyruvate-2-^{14}C

Nutritional state	Total radioactivity in glutamate	Avg % of total radioactivity in carbon atoms		Randomization in carbon 4
		no. 4	no. 5	
	μCi/mmole			%
Normal diet pair-fed (10) [1]	1.31	12.2	58.5	34
Oxythiamine-treated (7)	1.8	7.6	48	27

[1] Numbers in parentheses indicate number of animals used.

TABLE 3

Comparison of rates of gluconeogenesis in normal and thiamine-deficient rats

Exp. no.		Rate [1]
1	Thiamine-deficient	58.8
	Pair-fed control	52.15
2	Thiamine-deficient	49.78
	Pair-fed control	52.65
3 [2]	Thiamine-deficient	23.38
	Pair-fed control	24.4
4 [2]	Thiamine-deficient	23.08
	Pair-fed control	18.2

[1] mμCi ^{14}C incorporated into glucose/minute/g of rat weight from pyruvate-2-^{14}C.
[2] In experiments 3 and 4 the specific activity of the pyruvate was half that in experiments 1 and 2.

studies were carried out. The results are shown in table 3. No difference in the rates of gluconeogenesis between normal and thiamine-deficient liver is apparent. Radioactive isotope exchange and diffusion thus are not of consequence in the present interpretation. The data suggest that some sort of malfunction exists in thiamine deficiency affecting the selectivity for compounds entering the brain.

Histological examination of brain tissue from thiamine-deficient rats treated with trypan blue 24 hours before killing failed to show any trace of the dye. Since sensitivity of detection for radioisotopes far exceeds that of visual dye detection, we feel that the metabolic studies are showing the change in transport at a much earlier stage than can be shown using vital stains. Electron microscopic examination of thiamine-deficient tissue is now underway.

The blood-brain barrier is undoubtedly important in maintaining cerebral homeostasis and in the regulation of available metabolites. By responding to changes and needs it might also have an important role in the regulation of metabolic pathways and metabolic needs in the brain. In thiamine deficiency where the barrier is not functioning normally, pyruvic acid and perhaps other extracranial metabolites enter the brain. These metabolites may alter the metabolic pathways in this specialized tissue producing undesirable effects.

Whether the selectivity is a direct effect of thiamine cannot be answered here. It is known that glial cells have a high dependence on the hexose monophosphate pathway (19). It is tempting, therefore, to postulate that this alteration in selectivity reflects an alteration in the glial metabolism due to inadequate thiamine.

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Effect of Varying Calcium Intake on the Parameters of Calcium Metabolism in the Rat[1]

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ABSTRACT Kinetic tracer techniques providing the data for a compartmental analysis were supplemented with Ca balance measurements to obtain the rates of Ca absorption, endogenous fecal excretion, exchangeable space and accretion rates. The effect of low and high Ca diets was measured in terms of the above-measured parameters of skeletal metabolism in rats. Varying the Ca intake had no effect on the size of the compartments in the model used. Further, while the accretion rate of Ca into bone was not affected by the dietary Ca level, the rate of feedback of Ca from bone was quite sensitive to the level of Ca in the diet. The low Ca diet resulted in an appreciable increase in the resorption rate of Ca from bone and a very low rate of apposition of Ca into bone. However, the high Ca diet resulted in a minimal resorption rate (actually a negative rate) and a large increase in the net deposition of Ca in the skeleton. The use of a mathematical model proved to be a useful framework against which the effects of various Ca intakes could be measured quantitatively.

Although the kinetics of calcium metabolism are complex and difficult to characterize quantitatively, progress has been made by relating the biological system to a mathematical analog. The analog system is composed of a number of compartments, the sizes and transfer rates of which may be varied. While the mathematical compartments do not stand in a one-to-one relationship with readily identifiable biological components (for example, compartments represent "blood and soft tissues," "rapidly exchanging surface layer of bone," "slowly exchanging deep layers of bone"), nevertheless, the quantification of the calcium systems in terms of the above-mentioned parameters of compartment sizes and transfer rates has been very useful. Once it has been established that the model embodies the major characteristics of the physiological system, it can serve as an experimental base from which can be predicted the result of changes either in the input, or in the characteristics of the system itself.

In rats, abundant evidence has demonstrated that increasing the calcium content of the diet augments calcium retention in the form of skeletal mineral (1–4). In the present study, the effects of varying the Ca dietary intake in rats are interpreted in terms of the changes in the values of the rates of bone accretion and resorption as well as other calculated parameters of calcium metabolism.

To obtain a measure of the rate of intestinal absorption of Ca, resorption from bone, and apposition (net deposition) to bone, it was necessary to supplement the kinetic tracer technique with a classical calcium balance study. Since changes in bone turnover rate are the net result of bone formation and resorption, balance studies alone do not indicate which of the 2 factors is responsible for the change. Thus, both a kinetic tracer study and a balance study must be carried out simultaneously to provide the essential data. In addition to these studies, a whole-body counter was used to measure the whole-body retention of the injected tracer.

EXPERIMENTAL

Animals and diet. Fifteen male rats of the Sprague-Dawley strain, 81 days of age (285–309 g) were divided into 3 groups and fed either a low, medium or high Ca diet containing 1.9, 13.6 and 23.4 mg Ca/g food, respectively. (One rat fed the low Ca diet died accidentally before the completion of the study and two extra rats were added to the high Ca group making a total of

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seven). The animals were fed the diets for 3 weeks before starting the metabolic and tracer study, to assure equilibration. Calcium and phosphate salts were added to a standard calcium-deficient test diet.[3] All diets were identical except for the levels of Ca and P. The Ca/P ratio of all diets was 1.5. The amount of food consumed was measured as the difference between the amounts available to the rat before and after the experiment.

Balance study The stable Ca in the diet, plasma, urine and feces samples was determined by flame emission spectrophotometry (5).

Kinetic study. Carrier-free $^{85}SrCl_2$ (20 μCi) in saline at pH = 6 was injected intraperitoneally into each rat.

Blood samples (0.2–0.4 ml each) were taken from the tail of each rat at 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours after injection. This blood sampling procedure was designed to minimize excess blood loss, and is accurate and reproducible for serial sampling, as previously demonstrated (6, 7).

Urine and stool samples were collected over 24-hour intervals during the experimental period in specially designed individual metabolism cages (6, 7).

All samples were counted with a NaI (T1) scintillation counter, connected to a 400-channel pulse height analyzer. Samples were counted sufficiently long to assure better than ±3% accuracy.

In vivo whole-body retention of ^{85}Sr was measured by counting the rats in a whole-body counter immediately after injection and at the same intervals as the blood sampling up to 72 hours. The animals were counted in a fixed geometry by being placed in a glass tube in a whole-body counter designed for this purpose (6, 7). The detector was 7.62-cm (3-inch) NaI (T1) crystal connected to a 400-channel pulse height analyzer.

Compartmental analysis. The compartmental model used in this study and illustrated in figure 1 was previously formulated using ^{85}Sr and ^{47}Ca kinetic data obtained in studies in rats (6, 7) and in men (8, 9). The Berman SAAM program (10–12), converted for the CDC 6600 computer was used to perform the required

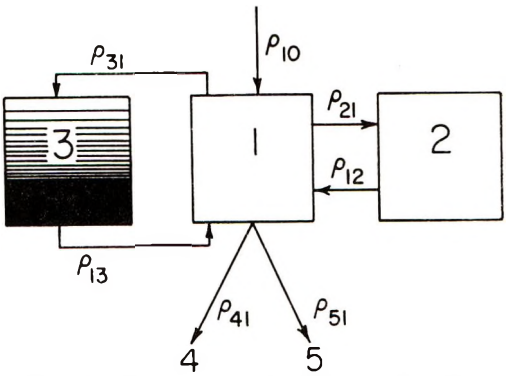

Fig. 1 Compartmental model of calcium kinetics. Compartments are designated as follows: 1) the physiological pool of calcium in isotopic equilibrium within minutes (plasma-extracellular-intracellular); 2) the physiological pool of calcium in isotopic equilibrium within hours (exchangeable bone); and 3) the calcium in "deep bone" or very slowly exchanging bone. The transfer rates, ρ, are designated as follows:

ρ_{10} = calcium intake rate
ρ_{12} = Ca flow rate into compartment 1 from exchangeable bone
ρ_{21} = Ca flow rate into exchangeable bone from compartment 1
ρ_{13} = rate of resorption and slow exchange from bone
ρ_{31} = rate of accretion into bone
ρ_{41} = urinary calcium excretion rate
ρ_{51} = fecal calcium excretion rate

calculations. This program, by an iterative technique obtains the "best" coefficients for a set of given differential equations describing the above model. This set of coefficients is then used to determine the parameters, the sizes of the compartments and the transfer constants between them. The flow rates (ρ) are then calculated from the product of the transfer constants and the sizes of the compartments.

Balance study calculations. The amount of stable calcium in the single rapidly turning-over compartment was assumed to be

[3] The percentage composition of the diet was: vitamin-free casein, 24; sucrose (analytical grade), 68; cottonseed oil, 5; Ca-free salt mixture, 3, and vitamin-fortification mixture. The Ca-free salt mixture contained: (in percent) magnesium chloride, 47.5; potassium citrate, 20.7; potassium chloride, 19.7; sodium chloride, 8.2; potassium sulfate, 2.1; ferric citrate, 1.6; potassium iodide, 0.04; sodium fluoride, 0.04; manganous sulfate, 0.02; and potassium alum, 0.008. The vitamin-fortification mixture included/kg of diet: (in grams) vitamin A conc (200,000 units/g), 0.099; vitamin D conc (400,000 units/g), 0.006; α-tocopherol, 0.110; ascorbic acid, 0.990; choline chloride, 1.650; inositol, 0.110; menadione, 0.050; p-aminobenzoic acid, 0.110; niacin, 0.099; riboflavin, 0.022; pyridoxine·HCl, 0.022; thiamine·HCl, 0.022; pantothenol, 0.112; and (in milligrams) biotin, 0.44; folic acid, 2.18; and vitamin B_{12}, 2.97.

TABLE 1

Computer-derived parameters for calcium metabolism

Diets [2]		Compartment size			Transfer rates			
					Urine [1]	Feces	Accretion	Flux
		1	2	1 + 2	ρ_{41}	ρ_{51}	ρ_{31}	$\rho_{12\cdot21}$
		mg			*mg/day*			
1	Low Ca	10.95 ± 1.01 [3]	50.47 ± 4.56	61.42	0.94 ± 0.96	3.05 ± 0.48	52.56 ± 2.88	92.88 ± 20.20
2	Medium Ca	14.21 ± 1.41	47.61 ± 3.40	61.82	1.33 ± 0.67	6.34 ± 0.46	55.20 ± 7.44	96.24 ± 15.10
3	High Ca	14.60 ± 1.34	51.50 ± 9.30	66.10	1.15 ± 0.22	9.96 ± 0.60	60.63 ± 4.40	103.7 ± 14.9
4	Comparison group	9.18 ± 0.39	49.68 ± 5.59	58.86	1.56 ± 0.15	9.62 ± 0.62	50.88 ± 2.14	91.90 ± 8.40

[1] Renal discrimination factor (Sr/Ca) of 10 was used to convert Sr data to milligrams of Ca.
[2] The Ca/P ratio of all diets was 1.5.
[3] SD.

of constant size, so that input of calcium into the pool is equal to egress of calcium from the pool. The net fecal Ca is equal to the difference between the total measured Ca in the stool and the endogenously excreted Ca. The resorption rate (R), therefore, is equal to the absorbed Ca (I) minus the Ca lost through urinary (U) and fecal excretion (F) and by uptake into bone (A) or

$$R = I - (U + F + A).$$

The difference between the measured intake (Ca in ingested food) and the net fecal Ca is equal to the amount of Ca absorbed across the gastrointestinal tract.

RESULTS

The values for the parameters of calcium kinetics (compartment sizes and transfer rates) as obtained by the computer for the compartmental model shown in figure 1 are presented in table 1. The compartments and the transfer rates of the compartmental model are defined in figure 1. The mean standard deviation of the various parameters of Ca metabolism of the 3 groups are shown. The overall reliability of the model as previously determined in a larger study of 40 rats is indicated by an average standard deviation for all the computed parameters of ±11% (6, 7).

For illustration, the computer-derived plasma and cumulative urine and feces curves are plotted along with the experimental data points of a represetnative rat (fig. 2). The computer-derived curves, it should be emphasized, are obtained by a simultaneous fitting of all the experimental data (plasma, urine, feces and whole-body count).

The sizes of compartments 1 and 2 do not differ significantly among the 3 groups fed the low, medium and high Ca diets (table 1). As a check on the analysis, the mean values of a previously studied group of Sprague-Dawley male rats fed a standard commercial laboratory ration [4] are included as group 4 (comparison group) in table 1. This latter group of rats was slightly older, but was fed approximately the same Ca and P levels (1.42% Ca and 0.96% P) as the rats in the medium Ca group in the

[4] Purina Chow Check Diet, Ralston Purina Company, St. Louis.

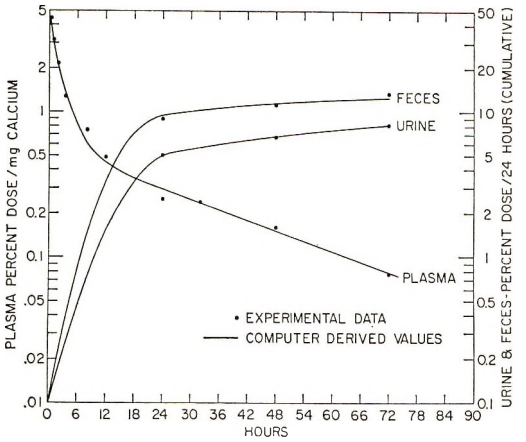

Fig. 2 CDC 6600 computer-derived fit to experimental plasma, feces and urine data; • experimental data; —— computer-derived values.

present study. Slightly lower values for the parameters would, therefore, be expected in the comparison group (6, 7). The reproducibility of the calculated parameters is obvious by comparing the 2 groups, and particularly by allowing for the slight age differences.

The accretion rate (ρ_{31}) or rate of movement of Ca into bone was calculated to range from 55.6 to 60.6 mg/day, and did not differ significantly among the 3 groups. While no difference was found in the rate of urinary excretion (ρ_{41}), the endogenous fecal excretion rate (ρ_{51}) increased with increasing levels of Ca in the diet. The urinary Ca values were derived from the Sr tracer data by using a previously derived factor of 10 (6, 7) to correct for the renal discrimination against Sr.

The various parameters used in calculating the resorption rate (ρ_{13}) via the calcium balance technique are shown in table 2. The Ca intake varied more than tenfold from the lowest group (22.3 mg/day) to the highest group (296.9 mg/day). The total Ca measured in the feces varied from 11.3 mg/day in the low Ca group to 226.9 mg/day in the high Ca group, or twenty-fold. The mean retention measured by whole-body counting was found to be 5% lower than when determined from the excretion data.

Although the absolute level of calcium absorbed increased with increased dietary calcium, the percentage absorption decreased from a high of 63.2% with the low Ca diet to 26.9% of the Ca intake in the high Ca diet group.

The resorption rate is the significant parameter that varies with dietary Ca in this study. The resorption rate is high in the low Ca group, while it is negative in the high Ca group. The apposition rate, or net rate of deposition of Ca in the skeleton (indicated in the last column of table 2) increased markedly with increasing dietary Ca intake.

The average body weights of the rats is also shown in table 2 over the 72-hour experimental period. There was no significant difference in the mean weights among the 3 groups. All groups ate approximately the same daily amount of the various diets over the 72-hour period. The average daily weight gain for the 3 groups over the third week of the diet, just preceding the tracer study, was 5.8, 5.8 and 7.1 g/day, respectively. The whole-body retention of the tracer ^{85}Sr at 72 hours after injection did not differ significantly among the 3 groups.

DISCUSSION

The model, while a simplification of the physiological system, is consistent mathematically and is compatible with the data. The close correspondence between the experimental data and the calculated values (fig. 2) indicates that the model represents the data well. Another test of the adequacy of the solution is the reproducibility of the calculated parameters (see groups 2 and 4).

The ability of the computer program to determine the degree of uncertainty of the parameter values of the model (average: SD = ±11%) permits still another way of assessing the reliability or uniqueness of the particular quantitative solution. For these reasons, the compartmental analysis serves as a very useful framework for quantitatively comparing the effects of different dietary Ca intakes on Ca kinetics.

In the steady state kinetic system, Ca is absorbed through the intestinal tract into the blood (compartment 1). Compartment 2 designates the physiological pool of Ca that comes into equilibrium with compartment 1 by 12 hours. Once compartment 1

TABLE 2

Calcium balance data

Diets [1]	Body wt	^{85}Sr retention at 72 hr	Daily Ca intake	Urine Ca	Total fecal Ca	Endogenous fecal Ca	Net fecal Ca [2]	Absorbed Ca [3]	Resorption rate [4]	Apposition rate [5]
	g	%	*mg/24 hr*							
1 Low Ca	290 ± 7 [6]	75.55 ± 1.20	22.3 ± 1.86	0.67 ± 0.04	11.3 ± 1.06	3.1 ± 0.48	8.2	14.1 (63.2%)	42.2 ±2.5	10.4
2 Medium Ca	309 ± 14.6	71.00 ± 2.09	158.8 ± 4.62	3.33 ± 0.27	108.2 ± 11.3	6.3 ± 0.46	101.9	56.9 (35.8%)	8.2 ±12.4	47.0
3 High Ca	285 ± 13.7	72.45 ± 1.12	296.9 ± 17.9	10.50 ± 0.65	226.9 ± 14.9	10.0 ± 0.60	216.9	80.0 (26.9%)	−18.0 ±13.5	78.6

[1] The Ca/P ratio of all diets was 1.5.
[2] Net fecal Ca = total fecal Ca − endogenous fecal Ca.
[3] Absorbed Ca = daily Ca intake − net fecal Ca.
[4] Resorption rate (ρ_{13}) = absorption (ρ_{10}) − accretion (ρ_{31}) + urinary excretion (ρ_{41}) + endogenous fecal excretion (ρ_{51})).
[5] Apposition rate = accretion rate (ρ_{31}) − resorption rate (ρ_{13}).
[6] SE.

and 2 reach equilibrium, Ca leaves this pool via excretion (ρ_{41} and ρ_{51}) and via passage into compartment 3 (ρ_{31}).

Calcium is deposited in compartment 3 in the calcifiable matrix of newly formed bone, and also by slow exchange into the already formed bone lattice. Ca in like manner leaves compartment 3 by resorption of bone and by slow exchange of Ca atoms from within bone. The processes of bone formation and resorption occur simultaneously and continuously in rats. The tracer (^{85}Sr) mixes very slowly with compartment 3 which is quite large and heterogeneous. For this reason compartment 3 is visualized as consisting of a large number of subcompartments or as an infinitely expanding compartment. Thus, while there is a constant feed-back of Ca, in the steady state, the amount of tracer feeding back from bone over the 72-hour period of the kinetic study is very small. Almost all of the Ca feeding back is nonlabeled with ^{85}Sr in this study; therefore ρ_{13} was considered to be zero in the computer solution. This is a justifiable assumption on the basis that with ρ_{13} equal to zero, the computer solution fits the data well. In further studies extending over longer periods of time, ρ_{13} becomes significant and setting it equal to zero results in a poor fit of the data. With the present technique ρ_{13} can be measured only indirectly, using data derived from calcium balance studies (table 2).

In terms of the "computer-derived" parameters, the most significant observation is that varying the Ca input to the system did not significantly alter the accretion rate (ρ_{31}) or the sizes of compartments 1 and 2. Even with the high Ca diet, the distribution pools of Ca in the body (compartments 1 and 2) did not increase nor did the rate of movement of Ca into bone (ρ_{31}). When these results are combined with the results from the Ca balance study, it is clear that the parameter that varies most with Ca intake is the resorption rate or more correctly, the rate of feedback from bone to blood (ρ_{13}). Thus the data indicate that the change in the net balance is due to the decrease in feedback from bone and not due to any change in the rate of movement of Ca into bone.

Ca absorbed across the intestinal tract enters the blood and rapidly leaves, since no increase in the level of plasma Ca could be detected, particularly under the high Ca diet. The percentage of the ingested Ca absorbed varies indirectly with the amount of ingested Ca. The greatest percentage absorption occurred in the group fed the low Ca diet. In all 3 groups the intercompartmental flux (ρ_{12}, ρ_{21}) was the same, indicating that the amount of Ca in the diet had no effect on the rate of mixing with the rapidly exchanging Ca pools.

In the steady-state condition, the level of plasma Ca is maintained constant and is regulated in part by the transfer of Ca between bone (the large Ca reservoir) and compartments 1 and 2. It is probable that compartment 2 consists mainly of rapidly exchanging Ca (62 mg), mostly in the more labile fraction of the skeleton. The physico-chemical exchange of Ca from this compartment is adequate to counter changes in the blood Ca level, which is about 2 mg in the rat. Further, the flux ($\rho_{12,\,21}$) is sufficiently rapid to quickly buffer these changes in the plasma Ca level.

The main regulation of plasma Ca, however, is slower acting and is mediated through the parathyroid hormone (PTH) which acts directly on the slowly exchanging bone compartment to stimulate bone resorption. The parathormone action now appears to be opposed by the action of thyrocalcitonin (TCT) which acts to inhibit bone resorption (14, 15). Thus both PTH and TCT participate in the physiological control of Ca metabolism (15).

The hypocalcemic thyrocalcitonin may be postulated as the mechanism to explain the observed action of the high Ca diet on the inhibition of bone resorption. Thus it is possible that the effect of high Ca intake in the present study, in decreasing bone resorption, may be mediated by TCT. With the low Ca intake, the Ca required to maintain the serum Ca could be supplied by the action of PTH on the bone resulting in the high resorption rate.

One result of this control system is that on a low Ca intake the resorption rate (ρ_{13}) is almost as large as the accretion rate (ρ_{13}) and so the apposition rate is very small. With the high Ca intake, the opposite result obtains and the difference between resorption and accretion is very large and thus the apposition rate is quite high (78.6 mg/day). This high apposition rate implies an increase in bone size or an increase in density of existing bone, or both.

An inherent weakness of the present study results from the necessity of calculating the resorption rate indirectly using balance data. The negative resorption rate, for example, with the high Ca intake is probably due in part to the inherent errors of the balance technique. In the balance technique, most errors involve small cumulative losses of feces and urine and incomplete measurement of intake which favors a positive balance (13). The variability in the balance measurements of the individual rats in the present study, even under carefully controlled conditions, yields a wide range of resorption rates. With the high Ca diet, for example, the standard error in the individually calculated resorption rates is ±13.5 mg/day. The variability of the calculated resorption rate with the low Ca diet is, of course, much smaller (±2.5 mg/day).

The fact that the whole-body retention of ^{85}Sr, measured at 72 hours, did not differ significantly among the groups is consistent with the explanation that the fraction of compartment 3 feeding back in compartment 1 is not labeled to any extent in the 72-hour period.

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