Metabolic Fate of Dietary Tannins in Chickens [1]

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ABSTRACT The metabolic fate of tannic acid and its degradation product, gallic acid, was studied using both paper and thin-layer chromatography (TLC) to isolate and identify characteristic metabolic end products in the urine of chickens after the oral administration of these substances. TLC revealed the presence of gallic acid, 4-O-methyl gallic acid and pyrogallol in the urine of hens fed either tannic acid or gallic acid. Tannic acid apparently was hydrolyzed to gallic acid and a large part of this material was O-methylated and excreted in the urine as 4-O-methyl gallic acid. This would explain the beneficial effect of methionine and choline in the diet of chicks fed tannic acid or natural tannins. Decarboxylation of gallic acid accounted for another metabolite, pyrogallol. Paper chromatograms revealed a fourth derivative, pyrocatechol, but the presence of this substance could not be positively identified using TLC.

Chang and Fuller (1) presented evidence that when grain sorghums containing relatively high levels of tannin were fed to young chicks, growth rate was retarded and liver lipids slightly elevated. Similar results were obtained by feeding a level of tannic acid (1% of the diet) equivalent to that occurring in the high tannin grain sorghum. Supplemental choline and methionine partially corrected the growth inhibition caused by tannic acid and completely remitted that which was caused by the high tannin grain sorghum. Booth et al. (2) reported that gallic acid depressed growth of rats and caused fatty livers. When choline and methionine were added to the diet at levels well above the requirement, the toxicity of gallic acid was completely alleviated. In addition to methionine and choline, Fuller et al. (3) observed that arginine (or ornithine) is also involved in the detoxication of tannic acid in chicks. The combination of methionine, choline and arginine (or ornithine) reduced the toxicity of tannic acid fed at the 1% level more than either one alone and completely alleviated the adverse effects of 0.5% tannic acid in a manner which suggested an additive effect of these nutrients.

The major metabolite in the urine of rats and rabbits ingesting tannic acid or gallic acid was found by Booth et al. (4) to be 4-O-methyl gallic acid. The source of methyl groups for the O-methylation of gallic acid involved principally choline and methionine.

The objective of this study was to determine the metabolic fate of tannic acid and its degradation product, gallic acid, in chickens.

EXPERIMENTAL PROCEDURE

Single-comb White Leghorn hens were housed in finishing batteries and fed a practical laying diet during the experimental period. Three hens were used for the paper chromatograms and 5 hens for the thin-layer chromatograms. They were colostomized according to the method of Dixon (5) to facilitate collection of urine. After a period of recovery, urine was collected from each hen to be used as a control. Each hen then received 0.5 g gallic acid or tannic acid [3] in a gelatin capsule followed by a second dose of 0.5 g in 12 hours. Urine collections were made for a 24-hour period following the administration of each compound. Hydrochloric acid was added to each collection bottle to retard oxidation of labile phenolic excretory products.

The method of Booth et al. (4) was used to separate phenolic substances from the urine. Each urine sample, containing 25% of a 20% sodium chloride solution, was extracted with three equal portions of ether,

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[3] Gallic acid, practical and tannic (digallic) acid, practical, Eastman Organic Chemicals, Distillation Products Industries (Division of Eastman Kodak Company), Rochester, New York.

and the extracts combined. The ether extract was then evaporated to dryness under partial vacuum and nitrogen. The residues were dissolved in 2 and 3 ml of acetone and subjected to chromatographic procedures as follows.

Paper chromatography. The acetone-soluble material was subjected to two-dimensional paper chromatography, using Whatman no. 1 filter paper. Chloroform, acetic acid and water (2:1:1) were used as the solvent system in the first direction and 20% potassium chloride in the second (4). The solvent front was allowed to migrate by the ascending method, approximately 30 to 35 cm in each direction. Color of the spots was developed by spraying with diazotized sulfanilic acid and sodium carbonate overspray. Spots were identified by comparing their R_F values and colors with those of known substances which had been spotted on the paper for that purpose.

Thin-layer chromatography. Smooth glass plates, 20 cm × 20 cm, were coated with silica gel G, 0.5 mm in thickness. The plates were allowed to air dry for at least 24 hours after pouring. The acetone soluble fraction was then spotted approximately 2 cm from the edge of the plate. All samples were applied with a microsyringe. Benzene, acetic acid and methanol (45:8:4) were used as the solvent system and all plates were developed by the ascending method in regular tanks according to Stahl (6). The plates were sprayed with diazotized sulfanilic acid and spots detected by observing under ultraviolet light. The R_F values and colors of the unknowns were compared with known compounds as done with the paper chromatography. Substances corresponding to known compounds were scraped from the plates and placed in elution tubes. The material was removed by two extractions with 10 ml of acetone. The acetone extracts were then concentrated to small volumes and rechromatographed together with known compounds.

RESULTS AND DISCUSSION

The results of the paper chromatographic separation of phenolic substances from urine of hens receiving tannic acid or gallic acid are presented in table 1. The R_F values shown are averages of values obtained for the urine of three hens analyzed individually. The R_F values of known compounds and the color of each after development are also presented. The 4-O-methyl gallic acid was prepared according to the method of Booth et al. (4). Five distinct components from urine of hens fed either tannic acid or gallic acid were detected. The first four corresponded in both R_F value and color to the known compounds — gallic acid, 4-O-methyl gallic acid, pyrogallol and pyrocatechol, respectively. The fifth substance had an R_F value of 0.79 and was orange after spraying with the color reagents. None of these substances were detected in the urine of control hens, indicating that they were all derivatives of

TABLE 1

Analysis by paper chromatography of urinary constituents of hens after ingestion of tannic acid or gallic acid

Sample	R_F value	Color [1]
Urine no. 1 (from hens fed gallic acid)		
Spot no. 1	0.64	Green
2	0.78	Red-orange
3	0.72	Brown
4	0.78	Pink
5	0.79	Orange
Urine no. 2 (from hens fed tannic acid)		
Spot no. 1	0.63	Green
2	0.73	Red-orange
3	0.68	Brown
4	0.78	Pink
5	0.77	Orange
Known compounds		
Gallic acid	0.63	Green
4-O-CH_3-gallate	0.80	Red-orange
Pyrogallol	0.73	Brown
Pyrocatechol	0.81	Pink

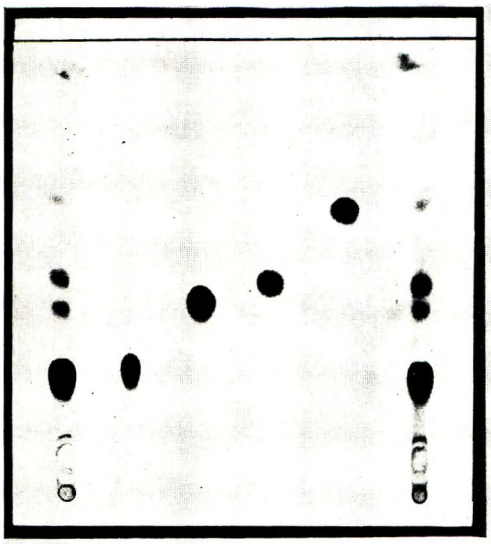
[1] After spraying with diazotized sulfanilic acid and sodium carbonate overspray.

Fig. 1 TLC separation of phenolic end products from urine of hens fed tannic acid. A, urine ether extract; B, gallic acid; C, pyrogallol; D, 4-O-methyl gallate; and E, pyrocatechol.

Fig. 2 TLC identification of substances eluted from TLC of figure 1. A, gallic acid; B, pyrogallol; C, 4-O-methyl gallate; A′, B′ and C′, phenolic components of urinary origin.

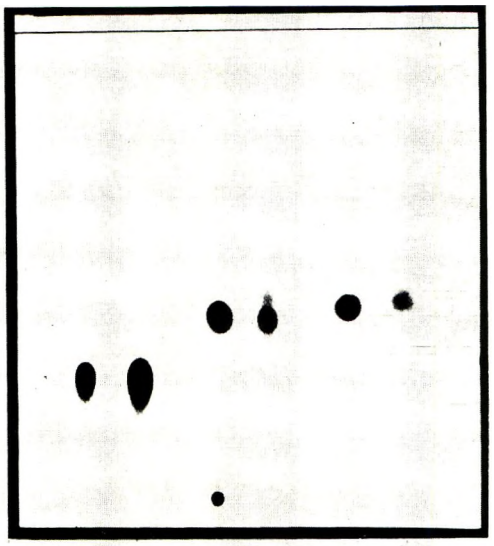
Fig. 3 TLC separation of phenolic end products from urine of hens fed gallic acid. A, urine ether extract; B, gallic acid; C, pyrogallol; and D, 4-O-methyl gallate.

Fig. 4 TLC identification of substances eluted from TLC of figure 3. A, gallic acid; B, pyrogallol; C, 4-O-methyl gallate; and A′, B′ and C′, phenolic components of urinary origin.



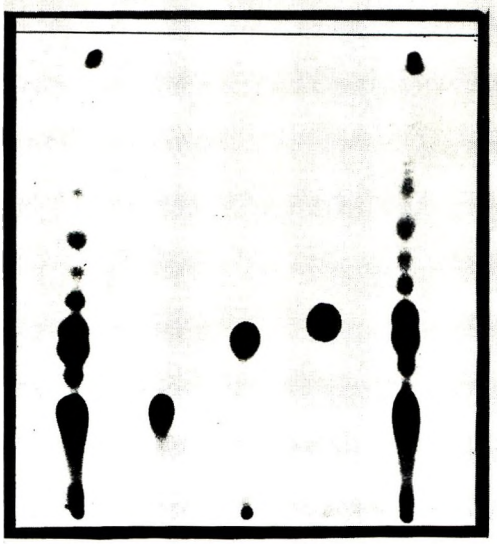
A B C D E A

1



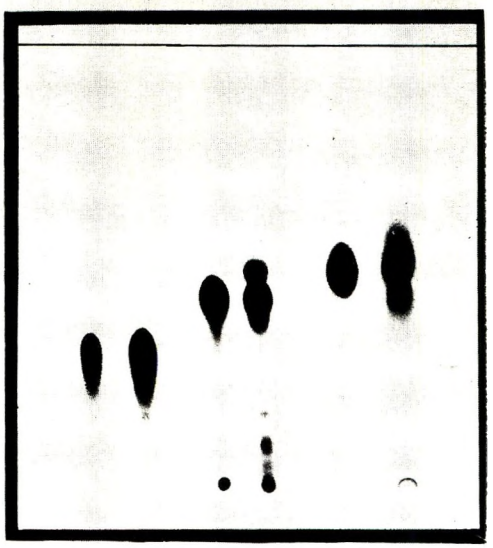
A A' B B' C C'

2



A B C D A

3



A A' B B' C C'

4

tannic acid or gallic acid or the direct results of feeding these substances.

Thin-layer chromatography (TLC) has not yet been used widely for the separation of phenolic substances; however, because of the greater sensitivity and the ease with which components can be recovered from thin-layer plates, it offers greater possibilities than does paper chromatography. Figure 1 shows the thin-layer separation and identification of phenolic substances present in urine of hens fed tannic acid. Similar chromatograms were prepared from the urine of each of five hens. Since there was no observable difference among hens, that shown in figure 1 was selected as typical. Six distinct components were detected, four of which corresponded in R_F values to gallic acid, pyrogallol, 4-*O*-methyl gallic acid and pyrocatechol. The color of the suspected pyrocatechol, however, did not correspond to known pyrocatechol, which was pink after spraying with diazotized sulfanilic acid and later turned dark; whereas, the urinary component was light orange and the color remained fixed upon standing.

Figure 2 shows the TLC identification of substances eluted from the chromatogram of figure 1 and rechromatographed. Spots A′, B′ and C′ corresponded to those of known gallic acid, pyrogallol and 4-*O*-methyl gallic acid (A, B and C), respectively, in both R_F values and color after spraying.

The separation of phenolic components from urine of hens fed gallic acid is presented in figure 3. The R_F value and color of three of these components corresponded to known gallic acid, pyrogallol and 4-*O*-methyl gallic acid as in figure 1. These materials were eluted and rechromatographed together with the corresponding known compounds, and the results are presented in figure 4. Spot A′ was identified as gallic acid. Two components were present in fractions B′ and C′. This was expected because of the condensed manner of the two materials in figure 3. Those were identified as pyrogallol and 4-*O*-methyl gallic acid. None of the other substances present in figures 1 and 3 were identified; however, two of them, the uppermost two in each figure, were also present in the control urine sample indicating that they were probably not derivatives of tannic acid or gallic acid. The other components present did not correspond to any of the several phenolic substances tested.

From these results, it appears that tannic acid is hydrolyzed to gallic acid in chickens and that a large part of this material is *O*-methylated and excreted as 4-*O*-methyl gallic acid as demonstrated for the rat and rabbit by Booth et al. (4). It was their conclusion that the source of methyl groups for the *O*-methylation of gallic acid probably involves both methionine and choline. Decarboxylation of gallic acid to form pyrogallol was also found to occur in chickens. Derivation of the pyrocatechol observed in the paper chromatograms and its failure to be positively identified in the TLC cannot be explained.

The role of arginine or ornithine in detoxifying tannic acid in chickens is unknown and may be either direct or indirect. Crowdle and Sherwin (7) observed that in the male cock, benzaldehyde, cinnamic acid and phenylpropionic acid were all oxidized to benzoic acid and then conjugated with ornithine to form α, δ-dibenzoyl ornithine or ornithuric acid; however, *p*-hydroxybenzoic acid was not conjugated with ornithine but was excreted unchanged in the urine. The authors so far have been unable to detect a conjugate involving gallic acid or a derivative of this compound and ornithine in the urine of chickens. The unknown components shown on the chromatograms of figures 1 and 3 contained no nitrogen as measured by the method of Cheronis and Entrikin (8), which would indicate that ornithine is probably not a component of either of these substances. Since a conjugate of ornithine and gallic acid, if existing, probably would not be very soluble in ether, 50 ml of urine from hens fed tannic acid were evaporated to dryness and the residue dissolved in hot methanol. The methanol soluble fraction was then subjected to TLC using the same procedure as for the urine ether extract. No additional substance was detected. The possibility of their existence is by no means eliminated, however, since exhaustive differentiating extraction and TLC procedures were not used.

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Vitamin E Activity of *N*-Methyl-β-Tocopheramine in the Rat Reproduction Assay

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ABSTRACT A derivative of β-tocopherol, *dl*,*N*-methyl-β-tocopheramine, was compared with *dl*,α-tocopherol for its vitamin E potency in the rat resorption-gestation bioassay. A relative potency of 88.7 ± 7.4% (confidence limits of 77.1 to 107.4%, $P = 0.05$) confirms the essentially equivalent activity of *dl*,*N*-methyl-β-tocopheramine and *dl*,α-tocopherol previously reported in the chick and also in the rat red cell hemolysis test.

Among a new group of amine derivatives of tocopherols recently prepared (1), three compounds have been found to have vitamin E activity comparable to that of α-tocopherol. In the rat red cell hemolysis test, *dl*,α-tocopheramine, *dl*,*N*-methyl-β-tocopheramine and *dl*,*N*-methyl-γ-tocopheramine had activity equal to or greater than that of *dl*-α-tocopheryl acetate (1). In the chick, these three amine derivatives had activity equal to that of α-tocopherol in preventing either exudative diathesis or encephalomalacia (2). Since these compounds do not appear to be converted in vivo to their corresponding tocopherols (1, 2), their vitamin E activity has important implications in considering structure-function relationships. Of special interest are the *N*-methyl-β- and γ-tocopheramines since these compounds have only two methyl groups on the chroman ring in contrast to the three methyl groups of α-tocopherol.

Although the red cell hemolysis assay has been shown to give results with tocopherols similar to those obtained by the classic rat reproduction assay (3), the latter procedure is still considered by many investigators to be the most definitive method for establishing vitamin E potency. Consequently, it was considered important to evaluate *N*-methyl-β- or *N*-methyl-γ-tocopheramine in the rat gestation-resorption bioassay.

EXPERIMENTAL

The assay was performed as described by Mason and Harris (4) with the modification that the pregnant mothers were killed on day 16 of gestation (5). Weanling female rats of the Sprague-Dawley strain from the National Institutes of Health colony were fed vitamin E-free diet R-11 (6) of the following composition (in percent): vitamin-free casein, 22; salt mixture, 6; stripped lard,[1] 5; vitamin mixture in sucrose, 2; DL-methionine, 0.3; and sucrose, 64.7. When the rats weighed 180 g (after 5 weeks), breeding was begun with males fed commercial pelleted ration. The day following positive mating (presence of sperm in the vaginal smear) was considered day one of gestation. Animals were caged individually in a room maintained at 24 ± 1° with a twelve-hour cycle of light and darkness.

The test compounds were given in three divided doses on days 6, 7 and 8 of pregnancy. The dose levels were spaced at a log interval = 0.1761 (7). *dl*-α-Tocopherol and *dl*-*N*-methyl-β-tocopheramine were in the form of gelatin beadlets.[2] Prior to the experiment, a re-analysis of these preparations (2) by the $FeCl_3$-bipyridyl reaction and by their ultraviolet absorption spectra indicated a content of 1.1 and 1.2% of α-tocopherol and *N*-methyl-β-tocopheramine, respectively. The beadlets were mixed with 10 g of food each evening and the entire supplement was consumed during the night. Special precautions were taken to prevent loss from spillage.

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

[2] Kindly supplied by Dr. O. Wiss, F. Hoffmann-La Roche and Co., Basel, Switzerland.

At autopsy, the number of living fetuses, dead fetuses and resorption sites were recorded. In the evaluation of results, the presence of two or more living fetuses was considered a positive response. The relative potency of *N*-methyl-β-tocopheramine was estimated from a graphic treatment of the data (7) plotted by the method of least squares. In addition, a computation of the potency was made using the regression equations as described by Bliss (7).

RESULTS

The initial dosage levels selected (0.53, 0.80 and 1.20 mg) were recognized as too high after about half the females had been tested. Consequently, two lower doses were introduced with the result that the planned 10 animals/treatment was not realized. The response is shown in table 1. Estimation of the relative potency of *dl*,*N*-methyl-β-tocopheramine, as compared with *dl*,α-tocopherol, by the graphic procedure gave a value of 91.8 ± 12.8% (SE). When computed from the regression equations, the relative potency was 88.7 ± 7.4%, with confidence limits of 77.1 to 107.4% ($P = 0.05$).

DISCUSSION

These results confirm the equivalent vitamin E potencies of *dl*,α-tocopherol and *dl*,*N*-methyl-β-tocopheramine found in assays with the chick (2). The higher activities of *N*-methyl-β- and *N*-methyl-γ-tocopheramines found by Schwieter et al. (1) in the red cell hemolysis test have not been apparent in our assays.[3] These studies together, however, show most importantly that the α-tocopherol structure with three methyl groups in the 5, 7 and 8 positions of the chroman nucleus is not essential for optimal vitamin E activity.

It has generally been considered that the rat fetal resorption test is the ultimate criterion for evaluating vitamin E activity. The inference has been that α-tocopherol has a high degree of specificity at the molecular level of fetal or placental metabolism inasmuch as no other compound had comparable activity. Although a variety of nontocopherol compounds have vitamin E activity in curing or preventing various deficiency symptoms in different species, only methylene blue (8) and *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) (9) have been demonstrated to be active in rat reproduction, but at relatively high dietary levels. Some workers have felt that the action of these compounds could have been indirect through a "sparing effect" on residual α-tocopherol in the tissues, even though in the study with DPPD the animals were carried through several generations without α-tocopherol. In the present case of *N*-methyl-β-tocopheramine, a "sparing

[3] Dr. U. Gloor of Hoffmann-La Roche Co., Basel, in a preliminary comparison of *dl*,*N*-methyl-γ-tocopheramine and *dl*,α-tocopheryl acetate in the rat resorption-gestation assay, found a ratio of activities of 0.9:1, respectively (personal communication).

TABLE 1

Response of rats in the vitamin E bioassay

Compound	Dose [1]	No. of rats	No. of positive responses [2]	Fertility	Avg/mother	
					Live	Dead or resorbed
	mg			%		
dl,α-Tocopherol	0.24	5	0	0	0	7.4
	0.36	6	2	33.3	1	10.2
	0.53	8	6	75.0	4.0	5.8
	0.80	6	6	100.0	8.2	1.3
	1.20 [3]	4	4	100.0	8.5	1.0
dl,*N*-Methyl-β-tocopheramine	0.24	5	0	0	0	10.6
	0.36	8	1	12.5	0.4	9.8
	0.53	7	5	71.4	4.4	4.3
	0.80	6	6	100.0	7.8	2.3
	1.20 [3]	6	6	100.0	7.7	2.6

[1] Total dose given in three divided portions.
[2] Two or more live fetuses. Four unsupplemented rats had no live fetuses and an average of 10 dead or resorbed.
[3] Not used in the calculation of potency.

effect" explanation is not plausible since the effective doses were equivalent to those of α-tocopherol.

How the *N*-methyl-tocopheramines function at the molecular level is not known at present. The available evidence indicates they are not converted to their corresponding tocopherols (2, 10). In addition to our preliminary report (2) that these compounds are demethylated to their tocopheramines, we have subsequently shown that in liver of rats dosed with *N*-methyl-β-tocopheramine there is about one-fourth as much β-tocopheramine as *N*-methyl-β-tocopheramine.[4] It is thus possible that the tocopheramines are the active forms of these compounds.

Søndergaard and Dam [5] have found that *dl*,*N*-methyl-γ-tocopheramine had 88% and *dl*,*N*,*N*-dimethyl-γ-tocopheramine had 65% of the activity of *dl*,α-tocopheryl acetate in preventing chick muscle dystrophy. It will be important to know if the dimethyl compound undergoes demethylation in the body. If the *N*,*N*-dimethyl tocopheramines are demethylated then this would lend support to the hypothesis that β- and γ-tocopheramines can replace α-tocopherol. The effects of *N*-methylation on blood and tissue levels of β- and γ-tocopheramines and other considerations which may explain the high biological activity of these compounds have been presented elsewhere (2, 10).

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[4] Bieri, J. G., and E. L. Prival, unpublished results.
[5] Personal communication.

An Accurate in vivo Technique for Measuring Bone Mineral Mass in Chickens [1]

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ABSTRACT The purpose of this study was to adapt the Cameron-Sorenson technique for measuring bone-mineral content, in vivo, in humans to chickens. Bone-mineral content is determined by measuring the transmission of a monoenergetic photon beam through the right tibia (3 cm proximal from the tibia-metatarsal joint) with a scintillation detector. The radioactive source is ^{125}I, and the mineral content is expressed as bone mass units. The correlation between bone mass units and ash weight per centimeter bone is 0.955 for a live chicken with no flesh equivalent present, and 0.934 when a flesh equivalent material is present. The bone mass determined on a thin cross section of the bone is representative of the total mineral content of the bone. The bone mass as determined by this system is reproducible within 3.2%. Repositioning and varying the chart speed did not have an effect on bone mass, and changing the time constant from 0.5 to 1.0 second produced no significant effect on bone mass. Bone weight and size are poorly correlated with bone mass units. There is a poor correlation between percent bone ash and bone mass units since bone moisture, protein, and fat content affect bone ash percentage but do not change bone mass units.

A need has existed for many years for an accurate and sensitive method of measuring mineral content of bone in vivo (1, 2). Bone ashing and photo densitometry have been useful, but both methods have serious limitations. Bone-ash determinations require that the animal be killed, and if the investigator wishes to follow the mineralization process over an extended period of time, it involves a large number of animals and, in a sense, not true replication. The X-ray technique lacks sensitivity in that a change in mineral content of about 30 to 60% is necessary to be radiographically apparent (3, 4). There are also differences in X-ray films and film development techniques which make the technique less reliable (5). Bone-seeking isotopes, such as ^{45}Ca, ^{32}P, and ^{85}Sr, have been used to study the relationship of the element to its effect upon and participation in the mineralization process or demineralization process, or both (6, 7). These kinetic studies are also very useful but, again, lack sensitivity and often give results of questionable interpretation.

Cameron and Sorenson (8) developed a technique for determining bone-mineral content by measuring the transmission of a monoenergetic photon beam (^{125}I or ^{241}Am) through the sample with a scintillation detector. This method was then adapted to bone-mineral measurements in chickens by Babcock et al.[2] and Babcock and Montilla (9).

It is the purpose of this paper to describe the basic principles and modifications which were applied for measuring in vivo, bone-mineral content with accuracy. This technique is capable of detecting small changes and has better reproducibility than most other in vivo techniques.

PROCEDURE

This method is similar to the X-ray techniques in that both involve the measurement of the transmission of photon beams through bone. The major advantages of the scintillation technique are: 1) the transmission of the photon beam is measured by directly counting photons, 2) the photon beam is monoenergetic and well collimated, and 3) the effects of overlying tissue are taken into account.

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[1] Supported in part by Biomedical Research Grant no. 67-86940-1 from the National Institutes of Health.

[2] Babcock, S. W., J. Montilla, E. C. Naber and M. L. Sunde 1965 *In vivo* measurement of bone-mineral content: Accuracy, reproducibility, and biologic significance. Federation Proc., 24: 566 (abstract).

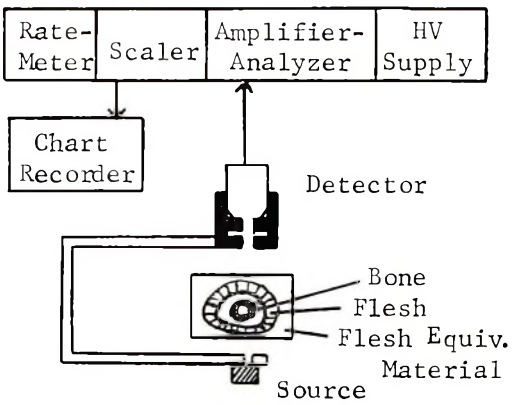

Fig. 1 Block diagram of the system.

According to Cameron and Sorenson (8), these factors eliminate errors resulting from the variability of X-ray films and development techniques, reduce the effects of scattered radiation, and reduce errors arising from the presence of tissue. The technique is also independent of both the thickness of overlying tissue and the orientation of the bone in the tissue.

A block diagram of the system is shown in figure 1. The basic components are a photon source, detector, preamplifier, high voltage supply, pulse analyzer, rate meter, and chart recorder. The photon source is ^{125}I (about 100 mCi) [3] and is prepared as described by Sorenson and Cameron (10). The photon source and detector are rigidly mounted and collimated to reduce the effects of scattered radiation. Scan speed is constant at 4.2 mm/second.

The equipment consists of a scintillation detector,[4] chart recorder [5] and a chicken-holding device. Figure 2 shows the equipment as used in making a typical measurement on a hen.

The chicken-holding device is designed to hold the chicken in a comfortable but

[3] Prepared by the Department of Radiology, University of Wisconsin, Madison.

[4] RIDL: 33-10B Single Channel Analyzer; 35-7B Linear Log Count Rate Meter; 40-12B High Voltage Power Supply; 49-28 Scaler. Radiation Instrument Development Laboratory, 4501 West North Avenue, Melrose Park, Illinois 60160.

[5] S-11A Westronics Chart Recorder, Westronics, Inc., 3605 McCart Street, Fort Worth, Texas 76110.

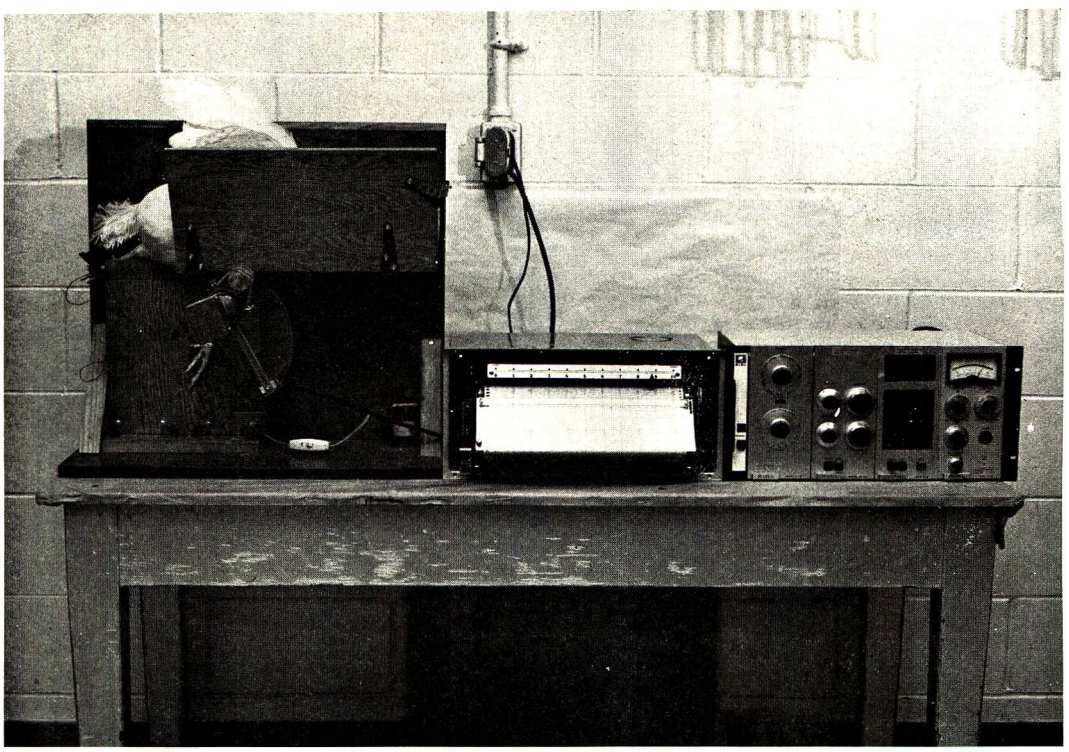

Fig. 2 Equipment for measuring bone mineral mass in chickens.

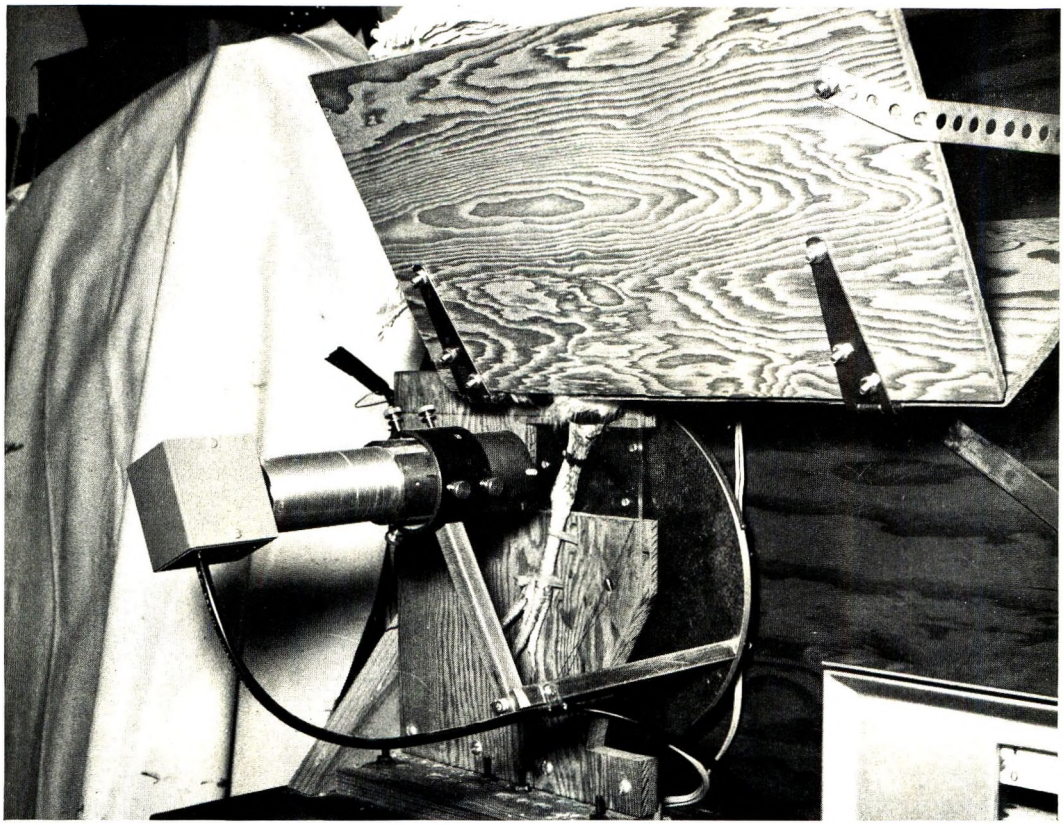

Fig. 3 Close-up of chicken-holding device showing the position of the chicken during a typical measurement.

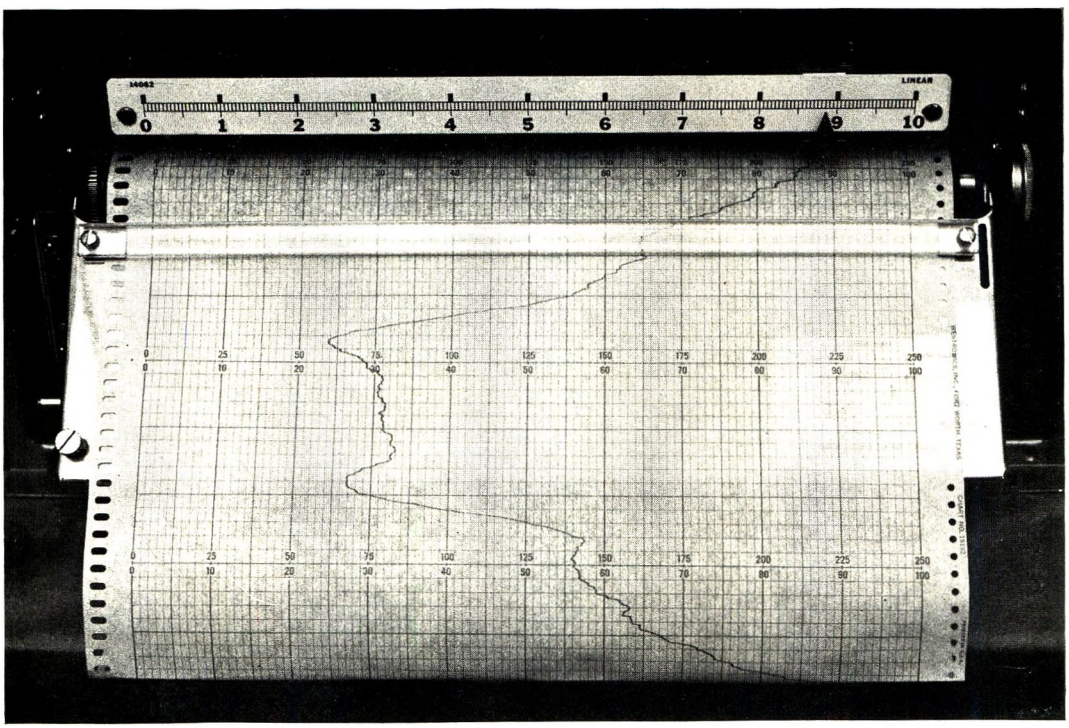

Fig. 4 Typical scan obtained during a bone-mineral mass measurement.

yet rigid position (fig. 3). If the chicken moves during a scan, it will affect the results; and the chicken must be repositioned and the scan done over. The measurements are taken across the tibia 3 cm proximal from the tibia-metatarsal joint. Before the chicken is put into the holder, the feathers in the scanning region are removed, and the 3-cm distance is marked with indelible ink to permit easy relocation for subsequent bone mass determinations.

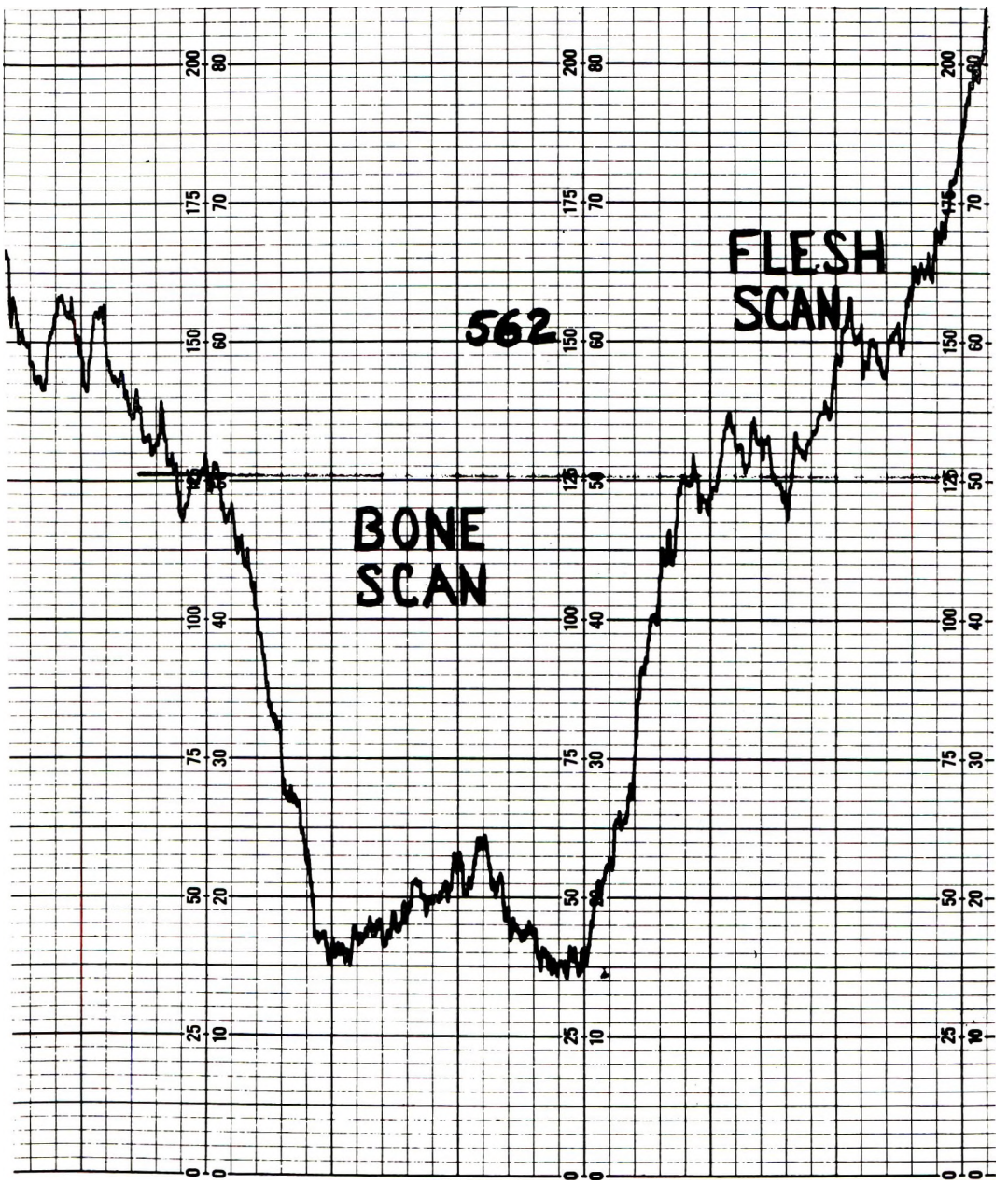

Fig 5A Close-up of a scan of hen no. 562 showing the flesh and bone portions with the I_0^* (base line) drawn at 504.

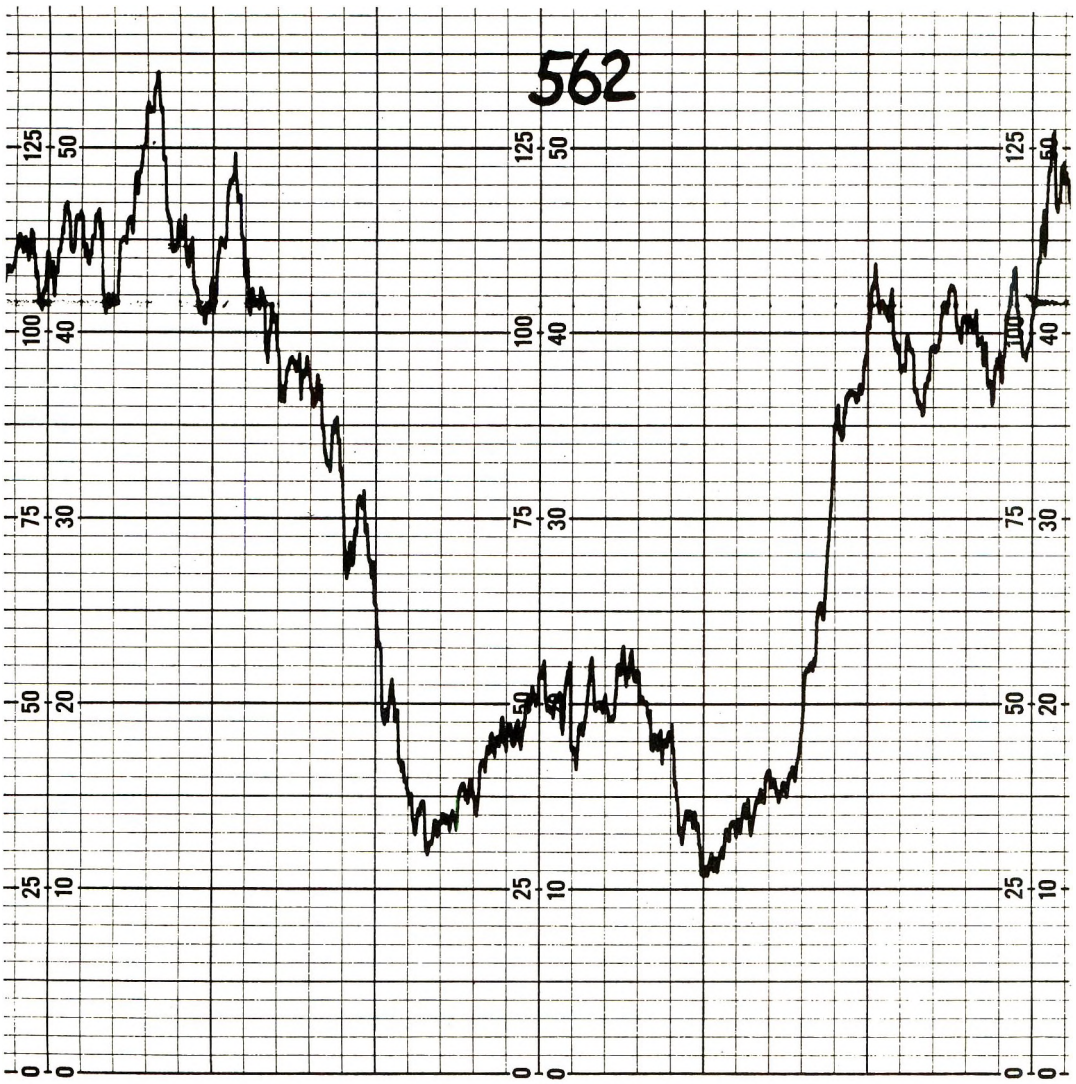

Fig. 5B Scan of same hen (no. 562) with flesh-equivalent material present.

The leg of the chicken is interposed in the photon beam in such a way that the beam scans transverse to the long axis of the tibia. At the start of the scan, the beam passes through air with only very little energy absorption; as the scan moves across the leg, first flesh then bone absorbs energy. As the beam scans the bone, there is a sharp increase in absorption. When the scan passes out of the bone, there is a decrease in absorption and then a leveling as the scan goes through the back flesh of the leg and into the air.

Figures 4 and 5 *A* and *B* show actual scans as they appear on the chart recorder paper; four such scans are taken for each bird per measurement. The scan in figure 5*B* is when the leg is surrounded by a flesh-equivalent material, such as water; water has about the same absorption coefficient as flesh. When the leg is scanned in water the only phases are a flesh phase and a bone phase; thus, the base line should be easier to determine and more accurate.

The bone mass is calculated as follows:

1. The base line (I_0^*) is calculated by first visually estimating the I_0^* from four

points at the start and finish of the scan. Then a value 80% of the estimated I_0^* is calculated and marked on the scan. The two data points most distal from the scan are excluded on each side leaving two points at the start and two points at the finish. These four points are obtained for each scan (the scan is replicated by four passes). The 16 values so obtained are averaged to obtain a final I_0^* which is drawn into each of those four scans.

2. Obtain the log value for each data point under I_0^*. Summation of these values gives Σ (the area beneath the curve).

3. Multiply the number of data points (N) by the logarithm of I_0^*. This gives A — the total area under I_0^*.

4. Subtract area Σ from area A. This value (A′) is the area between I_0^* and the curve and represents the undefined bone mass.

5. Multiply A′ by a factor which takes into account the relationship between the scan speed and the chart recorder speed (3.8992 at 20 cm/minute and 7.7401 at 10 cm/minute). This gives a value in "bone mass units" which is equivalent to those defined by Cameron and Sorenson (8). One "bone mass unit" is equivalent to 11.5 mg ashed bone in a one-cm segment of chicken bone.

Originally the values were calculated by hand, but now all data are entered on IBM cards and calculated by computer. Figure 6 shows a typical IBM card; spaces 1 to 3, experiment identification; spaces 4 to 8, date of measurement; 9 and 10, hen number; 11, scan number; 12 and 13, blank; 14 to 16, I_0^*; and then the data points are given with a space between each. After the last data point (usually on the second card), three dashes are punched which tell the computer it is the end of the scan. The computer then extracts log values, summates and calculates the areas and the individual bone mass values. It then averages the four scans per hen and calculates the standard deviation and the coefficient of variation.

Before and after each day's measurements, a hen standard is measured as a check on the equipment. This is an evacuated test tube (Vacutainer no. 3206, 5 ml size)[6] rigidly mounted on a plastic holder. It is scanned four times and gives a curve which is similar to the bone portion of a normal leg. The bone mass units are calculated by the same procedure as above which serves not only as a check within that day but also between days.

If this technique is to be useful, one of the first requirements is a high correlation between the bone mass units and the mineral content of the bone. To get a wide range of samples, 33 chickens of different ages and sexes were measured: 20-week-old pullets, 1-year-old layers, nonlayers, males, and five layers which were put on 0.11% Ca diet to produce demineralization and consequently values on the lower end of the range. The right legs of the live chickens were measured in "air," as shown in figure 3, in "water," killed, and the "bone" without flesh was measured in water. To facilitate measuring the same area after the flesh was removed, the bone was notched with a knife at the 3-cm mark after the bird was killed. The tibias were then measured (length and diameter at mark) and put in the desiccator for 24 hours. A 1-cm segment which had the notch in the center was cut out, measured for exact length ($\pm$ 0.01 cm), and weighed. The 1-cm segment and the rest of the bone were then ashed at 600° for 72 hours, cooled, and weighed. The percent ash, ash weight, and ash weight per centimeter bone were then calculated.

The reproducibility of our technique was also examined. The hen standard and five eighteen-month-old New Hampshire $\times$ Leghorn males were measured nine times in a 14-day period. The males had been fed a standard breeder diet for at least 10 months before the measurement.

The hen standard was measured in the normal position, removed and replaced several times to evaluate the effect of repositioning on reproducibility. Different combinations of chart speed and scaler response were also tested. The hen standard was also moved 5° in both directions to determine if the angle of the scan would significantly affect the resulting bone mass measurement. When the bone mass was computed, each position was calculated as a measurement; and then to get an overall

[6] Becton, Dickinson, and Company, Rutherford, New Jersey.

Fig. 6 Typical IBM card showing data for one scan: 1) Ash = experiment identification; 2) 03 = month; 3) 21 = day; 4) 7 = year; 5) 84 = hen number; 6) 1 = scan number; 7) 443 = I_0^*; and 8) 356 . . . = data points.

average and standard deviation, all the positions were calculated together as one measurement.

RESULTS AND DISCUSSION

The results of the bone-ash study are graphically shown in figures 7–11. The correlation coefficients were calculated by the least squares method and regression lines calculated from simultaneous equations for $y = a + bx$.

Figures 7, 8, and 9 show the high correlation between ash weight of the 1-cm segment and bone mass units. The correlation coefficients for "air" (live bird), "water" (live, leg in water), and "bone" (bare bone in water) are 0.955, 0.934, and 0.944, respectively. The fact that the correlation values for air and water, flesh intact, are not significantly different to that of bone (flesh removed) shows the presence of flesh is taken into account. Sorenson and Cameron [7] have suggested that composition of bone mineral may affect the accuracy. If this is true, higher correlation coefficients would have been obtained if only healthy, mature chickens had been measured instead of using birds from 20 weeks of age to 18 months of age and also birds that had been depleted of calcium.

The obvious advantage of using a flesh-equivalent material is fewer steps in calculation and hence, more accuracy. Automation has apparently overcome this objection, however, because the estimation of I_0^*, one of the most critical phases of the operation, was not improved by the use of the flesh-equivalent material.

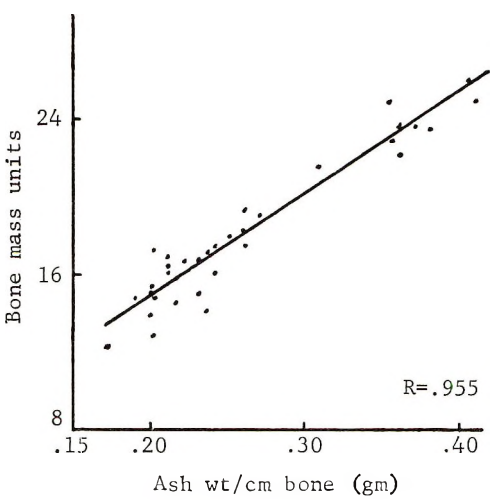

Fig. 7 Correlation between bone mass units and ash weight per centimeter bone when measured in air.

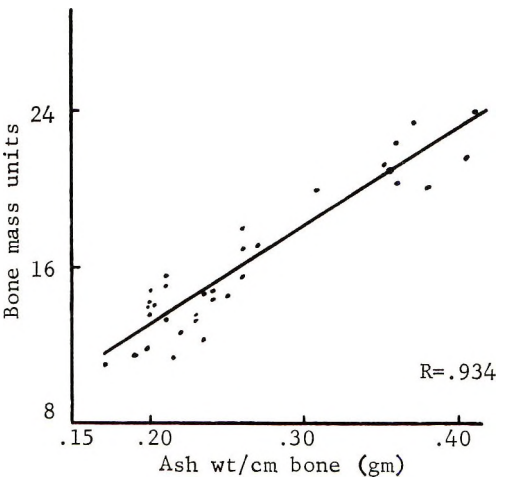

Fig. 8 Correlation between bone mass units and ash weight per centimeter bone when measured in water.

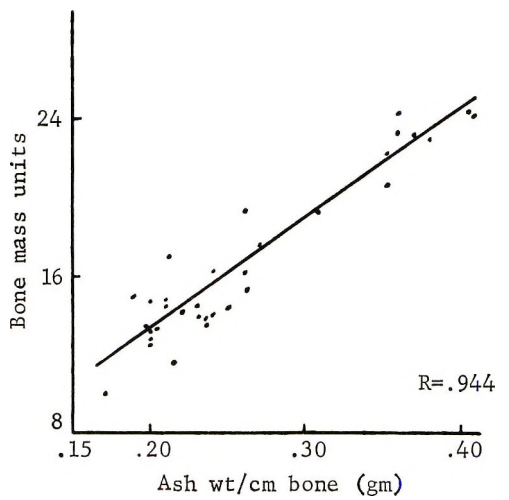

Fig. 9 Correlation between bone mass units and ash weight per centimeter bone when bone (flesh removed) is measured in water.

Similar correlation coefficients were observed when the ash weight per centimeter of the whole bone was compared with bone mass units. The respective coefficients for air, water, and bone are 0.938, 0.894, and

[7] Sorenson, J. A., and J. R. Cameron 1968 Personal communication.

0.924. These values are slightly lower than those of the 1-cm segment, but they do follow the same pattern in that air has the highest correlation and water the lowest. The differences are not statistically significant.

Taylor and Moore (11) showed that whereas actual cortical ash weight fell over 50% for hens on a Ca-deficient diet, the percentage ash was only slightly reduced; thus, they concluded that percent ash without reference to ash weight is a poor measurement for bone mineralization. Significantly lower correlations (fig. 10) were observed between percent ash and bone mass units, for both the 1-cm segment ($r = 0.816$) and the whole bone ($r = 0.638$). This is to be expected because percent bone ash expresses the ratio of bone mineral to the total bone tissue including protein, fat, and water; and this is not necessarily related to the total mineral content. Percent ash values ranged from 34.8 to 56.1% for the 1-cm segment and 23.0 to 39.3% for the total bone. These values are lower than those cited by Ewing (12), but those values are for normal hens ashed on a fat-free basis; whereas our values represent birds from a wide range of physiological conditions and were obtained on a wet-weight basis. In this study, the tibias were cleaned (flesh removed), then put in a desiccator for 24 hours, weighed, and ashed. Although Montilla [8] extracted the fat and obtained higher percent ash (average = 65% ash), the correlations of percent ash for a 1-cm segment to bone mass units were 0.667 and 0.680 for two different ash studies. These values were significantly lower than the correlation value obtained in this study. Both studies support existing evidence that percent ash is a poor indicator of bone mineralization when used without reference to ash weight. One would expect lower ash values using the present procedure than when the fat and water were removed prior to ashing.

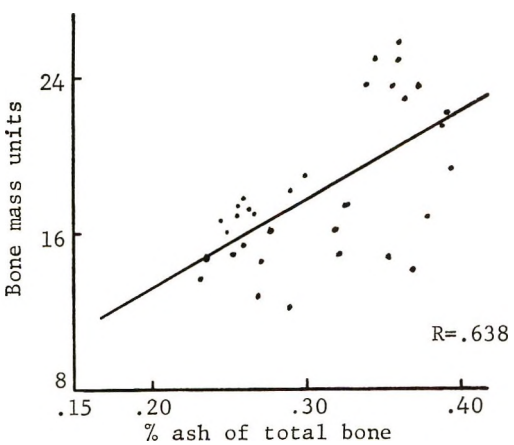

Fig. 10 Correlation between bone mass units and percent ash of total bone (measured in air).

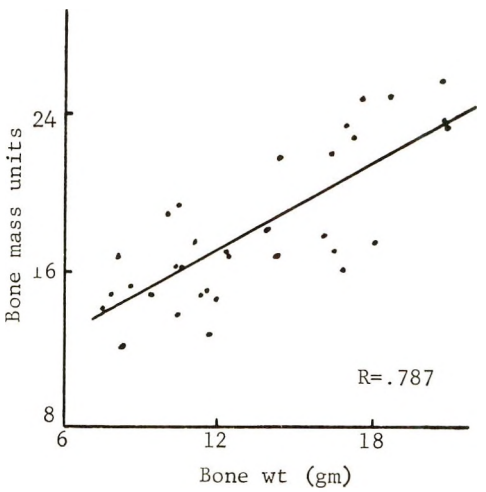

Fig. 11 Correlation between bone mass units and bone weight (measured in air).

Figure 11 illustrates the low correlation between wet bone weight (bone removed, put into desiccator for 24 hours, and then weighed) and bone mass units ($r = 0.787$). Figure 12 shows an even lower correlation ($r = 0.551$) between the diameter of the bone (largest diameter at mark where scanned) and bone mass units. Therefore, two bones which may look and weigh the same may give different bone-mass values depending on their actual mineral content. Thus, this system provides a means of determining bone-mineral mass and does not give accurate information as to the volume, shape, and internal porosity of the bone. Since volume is not accurately measured, bone density cannot be determined (both mass and volume are necessary to calculate density);

[8] Montilla, J. 1965 *In vivo* bone mineral measurements in poultry and the application in calcium metabolism. Master's Thesis, University of Wisconsin.

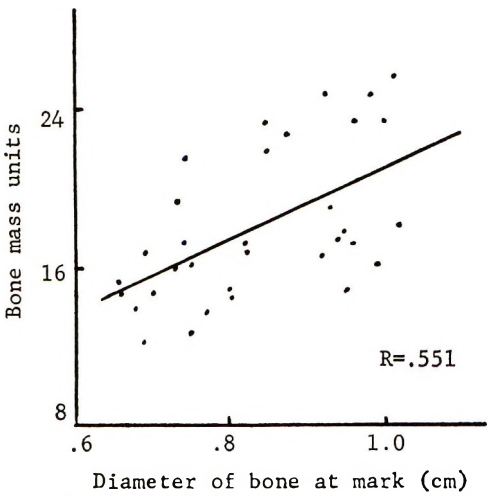

Fig. 12 Correlation between bone mass units and diameter of bone (measured in air).

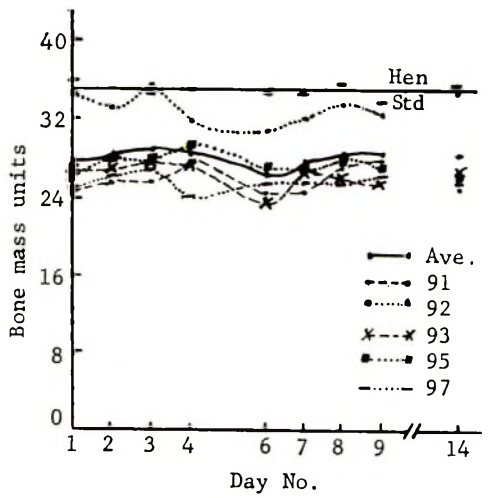

Fig. 13 Reproducibility of hen standard and male chickens (measured in air).

thus, it would be incorrect to refer to this technique as a bone-density measurement.

The results of the reproducibility study with the hen standard and five males are shown in figure 13. When the hen standards for the 9 days were calculated, the bone mass value equals 35.1 units with a coefficient of variation of 2.6%. It was thought that the mature males' bone mass would be constant throughout the test period. However, the units varied in a cyclic manner from day to day. The fluctuations may be due in part to variations in daily feed intake. To reduce the effect of daily variation, 2 days' values (such as day 1 and 2, 2 and 3, etc.) were averaged together. Although this reduced the size of the fluctuation, the bone mass still followed a rhythmic pattern which may suggest the small fluctuations that were detected may be important in short-term mobilization phenomenon for mineral balance, or ionic balance, or both.

Repositioning the hen standard in the second reproducibility study resulted in no change in the bone mass units. The average of 31 scans repositioned 10 times in the normal position (hen standard perpendicular to the arc of the scan) is 36.5. The time response was held constant at 1.0 second, and the chart speed at 10 cm/minute. When the scans were analyzed, it was found that the coefficient of variation was the same between positions as within a position (3.2%). Moving the hen standard 5° to the right and left of the normal position gave values of 36.1 and 36.3 which are within the 3.2% variation of the average; therefore, repositioning of the hen standard did not affect the bone-mass values, and the results are reproducible within 3.2%.

Cameron and Sorenson (13) reported that when the time constant is increased from 0.5 to 1.0 second, the measured bone mineral decreases by about 2%. Normally, our measurements are taken with a time constant of 1.0 second because the scans are much smoother, and the data points are easier to read. Measurements with a time constant of 0.5 second gave an average bone mass value of 37.7 compared with 36.5 at 1.0 second; a decrease of 3.2% in bone mineral content by using a 1.0-second time constant. Scan speed is also a very important factor in these measurements; however, the speed was held constant at 4.2 mm/second.

The effect of chart speed was also studied since the hen standard is usually measured at a chart speed of 10 cm/minute and the chickens at 20 cm/minute. When the hen standard (at 20 cm/minute) is repositioned three times, average bone mass was 35.5; with a coefficient of variation of 2.8%. Since these values are essentially the same as those at 10 cm/min-

ute, the chart speed has no effect on bone mass measurement.

Babcock et al.[9] and Babcock and Montilla (9) originally adapted this technique to chickens and did experiments similar to those reported here. Since then, new equipment and modifications in calculating and the chicken-holding device have made it necessary to repeat some of their work. The results described in the above report, although of a more preliminary nature, are very similar to the ones reported here.

The successful application of this technique will allow nutritionists and physiologists to approach many old problems in a new way. The effect a particular nutrient has on the mineralization process or demineralization process, or both, may be followed accurately and for any period of time without killing the animal. The effect hormones and related compounds have on growth, egg production, osteoporosis, and other physiological phenomena related to the skeleton may also be examined.

ACKNOWLEDGMENT

We express our thanks to James Sorenson and Dr. John Cameron for their technical advice and for supplying the ^{125}I sources used in this study, and to Dr. George Barr and Leo Hanson for their help in preparing the computer program and the use of the computers.

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[9] See footnote 2.

Catabolism of ^{14}C-Thiazole-labeled Oxythiamine and Its Effects on Thiamine Catabolism in the Rat [1]

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ABSTRACT The metabolism of oxythiamine and its effects on thiamine metabolism were studied. Three groups of six rats each were injected daily with 40 μg ^{14}C-thiamine, 40 μg ^{14}C-thiamine + 80 μg oxythiamine, or 40 μg thiamine + 80 μg ^{14}C-oxythiamine. Injections were continued for 8 to 20 weeks after which time the urinary excretions of thiamine and oxythiamine metabolites were studied. Under these conditions oxythiamine was less susceptible to destruction by opening of the thiazole ring than was thiamine. Oxythiamine was poorly retained by the rat tissue but enhanced the excretion of thiamine. Injection of somewhat higher oxythiamine-to-thiamine ratios (4:1, 6:1) resulted in a decrease in the amount of $^{14}CO_2$ generated from thiamine and the urinary excretion of thiamine and its metabolites. Higher ratios (8:1, 10:1) had less effect. Oxythiamine yielded only about half as many urinary metabolites as thiamine. One product of oxythiamine metabolism appears to be thiamine itself but the amount converted is extremely low and is of no apparent benefit to the rat.

Although much attention has been paid to the metabolic effects of oxythiamine administration (1–7) little has been directed toward the metabolic fate of the oxythiamine molecule itself. We are here reporting studies of the metabolism of this compound as well as studies of the effect on the metabolism of thiamine.

MATERIALS AND METHODS

The method of Rydon (8) was used to synthesize 2-^{14}C-thiazole-labeled oxythiamine from ^{14}2-C-thiazole-labeled thiamine. The latter compound [4] had a specific activity of 26.7 mCi/mmole and since it was diluted 50-fold with nonlabeled thiamine for the synthesis, the resulting ^{14}C-oxythiamine had a specific activity of 0.53 mCi/mmole.

The purity of the commercial ^{14}C-thiamine was checked and monitored by thin-layer chromatography. The identity and purity of the synthesized ^{14}C-oxythiamine was established by melting point, ultraviolet spectrum, and thin-layer chromatography. In some experiments, the ^{14}C-oxythiamine was exhaustively purified by chromatography on Amberlite CG-50 resin which serves to remove all traces of thiamine. The elution mixture consisted of pyridine–acetic acid–water (75:10:915, by volume).

Eighteen female weanling rats of the Sprague-Dawley strain were divided into three groups of six rats, and each group was housed in pairs in wire-screen stainless steel metabolic cages constructed to permit the separate collection of urine and feces. The rats were fed the thiamine-deficient diet described by Neal and Pearson (9) and were injected intraperitoneally daily with 40 μg thiamine and/or oxythiamine as follows: group 1, 40 μg ^{14}C-thiamine (1.2×10^5 cpm); and group 2, 40 μg ^{14}C-thiamine + 80 μg unlabeled oxythiamine (1.2×10^5 cpm); and group 3, 40 μg thiamine + 80 μg ^{14}C-oxythiamine (1.4×10^5 cpm). Under these experimental conditions, the amount of oxythiamine injected does not produce thiamine deficiency. The daily injections were continued for 8 to 20 weeks. Twenty-four-hour urine samples were collected in glass bottles containing a few crystals of thymol and 2 ml of 2 M acetic acid. At the end of each 24-hour collection period, the urine of each group (3 samples) was pooled separately, and stored at −20° until ana-

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[1] Supported by Public Health Service Grant no. AM-07709. Reprint requests should be addressed to Dr. Pearson.

[2] A portion of these data was taken from a thesis submitted by M. R. Ariaey-Nejad to the graduate faculty of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

[3] Present address: Iranian Army Nutrition Committee, Army Medical Department, Tehran, Iran.

[4] Nuclear-Chicago Corporation, Chicago.

lyzed. The quantitative data presented here were obtained only after a long period of injection (at least 8 weeks). The rats were fed diet and water ad libitum throughout the experiment.

Isolation of radioactive metabolites from urine and feces was accomplished by procedures already described in detail (9-11). Briefly, this entailed the adsorption of the metabolites on activated charcoal, recovery of the charcoal by centrifugation, and elution of the metabolites with a pyridine–ethanol–water mixture (75: 10: 915, by volume). The eluates were reduced in volume under vacuum, the pH adjusted to 5.5 and chromatography was performed on columns of Amberlite CG-50 (1.0 × 40 cm; 200 to 400 mesh, H^+ form). Metabolites were first eluted with 200 ml of water and then with 300 ml of the pyridine–acetic acid–water mixture. Five-milliliter fractions were collected and 0.25 to 0.5 ml of each fraction was plated on an aluminum planchet, dried under an infrared dryer,[5] and counted in a Nuclear-Chicago Model 8703 gas flow counter equipped with an automatic sample changer. No attempts were made to correct for internal absorption during this screening procedure.

The radioactivity of pooled urine samples, of tissues and feces extracts and of radioactive peaks obtained from column chromatography were measured in a Nuclear-Chicago liquid scintillation counter (Model no. 6801). These results were corrected for quenching by use of an internal standard.

For respiratory studies, the rats were placed in a plastic respiration chamber and the expired CO_2 was collected by drawing expired air first through a column of Drierite and then through two successive chambers, each of which contained 300 ml of a mixture of ethanolamine and ethylene glycol monoethyl ether (1:3, by volume). At the end of the collection period (8 to 24 hours) the trapping solutions were pooled and duplicate aliquots were taken for scintillation counting.

The extraction procedure described by Balaghi and Pearson (10) was used for tissues and feces except that 0.2 N HCl was used instead of 0.2 N H_2SO_4. When necessary, the feces extract was treated with acid-washed charcoal to concentrate radioactive metabolites prior to column chromatography.

In previous studies of this nature paper chromatography was used for further separation of the radioactive metabolites obtained by column chromatography (9–11). Because of better resolution and economy of time, thin-layer chromatography was used in these studies. Plates of 500 μ thickness were prepared using Microcrystalline Cellulose (MN-Cellulose Powder 400).[6] The solvent system used consisted of: *n*-propanol–water–1 M acetate buffer pH 5.0 (70: 20:10, by volume). For preparation of autoradiograms, the dried thin-layer chromatograms were marked in all corners with radioactive ink and then placed in contact with Eastman No-Screen X-ray film in a 20.3 × 25.4 cm Eastman Kodak X-ray film exposure holder. One- to two-week exposures were used depending upon the radioactivity. The films were then developed and fixed in the conventional manner.

Of necessity, some experimental procedures will be detailed in the following sections. Also, for the purpose of brevity 2-^{14}C-thiazole-labeled thiamine and 2-^{14}C-thiazole-labeled oxythiamine will be referred to as ^{14}C-thiamine and ^{14}C-oxythiamine, respectively, throughout the remainder of the discussion.

RESULTS AND DISCUSSION

On day 56 of the experiment the average weights in grams with standard deviations of the six rats in each group were as follows: group 1, 191 ± 20.5; group 2, 197 ± 12.4; and group 3, 214 ± 18.7. These differences were not significant. At this point, the rats in groups 1 and 3 were killed. Group 2 was maintained on its usual regimen (i.e., 40 μg ^{14}C-thiamine + 80 μg oxythiamine) for an additional 12 weeks to permit the collection of additional urine samples.

The excretion of radioactivity in the urine, feces and respiratory CO_2 was

[5] In later studies this treatment was found to volatilize some radioactivity and thereafter drying at a temperature of 37° was used.

[6] Macherey-Negal Company, Duren, Germany.

studied quantitatively in all three groups after 7 weeks of daily injections. These excretions calculated as percentage of the daily dose of radioactivity are seen in table 1.

More than half of the radioactivity excreted by each group appeared in the urine. Oxythiamine significantly depressed the excretion of total thiamine metabolites in the urine (group 1 vs. group 2). About 30% of the radioactivity of ^{14}C-thiamine-injected rats appeared in the feces as compared with 37% of the injected ^{14}C-oxythiamine. The mean $^{14}CO_2$ excretion (as percentage of the daily dose of radioactivity) was much greater in group 1 (^{14}C-thiamine alone) than in group 3 (thiamine + ^{14}C-oxythiamine). Group 2 (^{14}C-thiamine + oxythiamine) showed the greatest yield of $^{14}CO_2$. Thus, although oxythiamine is degraded at a much lower rate via this pathway it may enhance the destruction of thiamine via this route.

Although these data suggest that thiamine and oxythiamine are treated similarly, tissue analysis indicated that oxythiamine was poorly retained. In table 2 the carcass and liver radioactivities at autopsy of groups 1, 2 and 3 are expressed in terms of the daily total injected radioactivity. Both carcasses and the livers of group 1 (^{14}C-thiamine alone) retained approximately six times more radioactivity than did those of group 3 which received ^{14}C-oxythiamine + thiamine. These findings point up the shortcomings of balance studies of the type reported in table 1, in the estimation of the retention of a nutrient. Group 2 which was maintained on its regimen for 20 weeks, retained somewhat more carcass radioactivity than did group 1 but the time difference and limited data preclude assigning statistical significance to this observation. The finding that ^{14}C-oxythiamine was poorly retained in the tissues is compatible with its high concen-

TABLE 1

A comparison of the percentage of injected radioactivity excreted as $^{14}CO_2$, in urine and in the feces

Group	Daily injection	% of daily injected radioactivity excreted			
		$^{14}CO_2$	Urine	Feces	Total
1	^{14}C-thiamine	7.1 [1]	61 ±4.5 [2]	31 [3]	99
2	^{14}C-thiamine + oxythiamine	9.6 ± 0.9	54.6 ± 6.6	29.4	94
3	Thiamine + ^{14}C-oxythiamine	1.9 [1]	60.1 ± 9.9	37.1	99

[1] These CO_2 data represent the means of two consecutive 24-hour CO_2 collections from a single rat from each group. In group 2, single collections from each of six rats are averaged.
[2] These urinary data represent the means and standard deviation of 10 daily measurements of urinary radioactivity in pooled urine samples of each group. The difference in urinary radioactivity between groups 1 and 2 was significant ($P < 0.05$).
[3] To obtain these data, a 20-day group feces collection was extracted, counted, and a mean daily value calculated.

TABLE 2

Retention of ^{14}C activity in rat carcass and liver in response to three treatments [1]

Group	Compound injected	No. of weeks of daily injections	No. of rats	% of daily injected radioactivity found at autopsy	
				Carcass [2]	Liver
1	^{14}C-thiamine	8	6	602(550–747) [3]	155(135–187)
2	^{14}C-thiamine + oxythiamine	20	3	721(700–735)	—
3	^{14}C-oxythiamine + thiamine	8	6	90(72–112)	27(22–30)

[1] Slightly differing periods of injection required expression of these data in terms of some common base, i.e., in terms of total daily injected radioactivity. In groups 1 and 2 this was 1.2×10^5 cpm/rat/day. In group 3 this was 1.4×10^5 cpm/rat/day. Sample calculation: a rat in group 1 was found to have a total carcass radioactivity of 7.5×10^5 cpm. Thus, the percent radioactivity retained was calculated as $(7.5 \times 10^5 \text{ cpm} \div 1.2 \times 10^3 \text{ cpm}) \times 100 = 625\%$ of daily dose.
[2] The whole carcass (excluding heart, liver, kidney and brain) was analyzed for radioactivity.
[3] Figures in parentheses indicate the range of values.

tration in urine (see later) and is probably related to its limited phosphorylation (12).

The apparent effect of oxythiamine on the generation of $^{14}CO_2$ from thiamine was examined in more detail by altering the oxythiamine-to-^{14}C-thiamine ratios in a pair of rats selected from group 2. After an initial 24-hour CO_2 collection under the prevailing regimen (80 μg oxythiamine + 40 μg ^{14}C-thiamine), this oxythiamine ratio was increased in the same pair of rats on consecutive days to 4, 6, 8 and 10 with the thiamine dosage being held constant. As seen in figure 1, $^{14}CO_2$ production was first

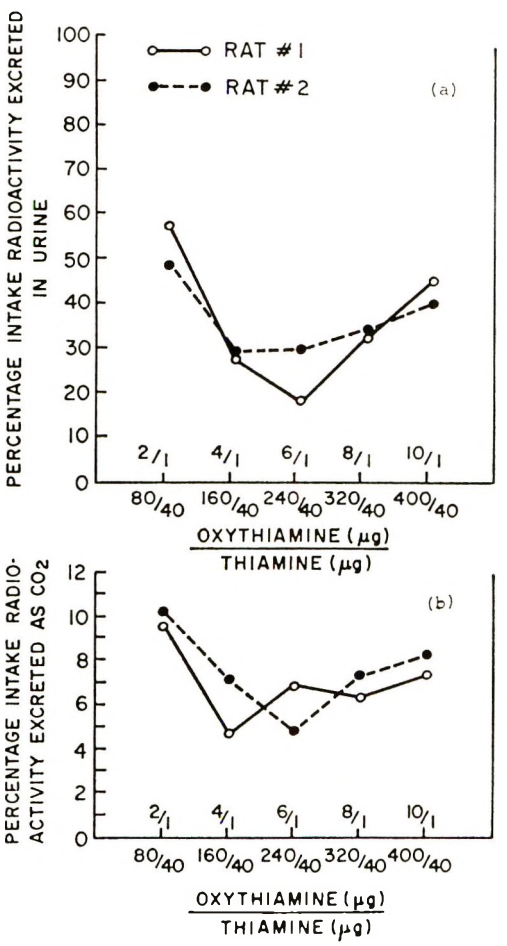

Fig. 1 Percentage of injected radioactivity appearing in urine and expired CO_2 of two rats receiving on consecutive days various ratios of oxythiamine to ^{14}C-thiamine (2:1, 4:1, 6:1, 8:1 and 10:1).

decreased and then returned to "normal" as the ratio increased. A similar pattern of radioactivity was found in the urine.

It was then necessary to determine if the observed changes were a function of dosage or of time. Accordingly, the same experiment was repeated except that a separate rat was used for each oxythiamine-to-^{14}C-thiamine ratio greater than 2:1. Thus, each rat (randomly selected from group 2) first received the regular 2:1 injection and CO_2 was collected for 24 hours. Then, the same rat was injected with a solution containing a different ratio of oxythiamine to ^{14}C-thiamine and CO_2 was again collected for 24 hours. It is evident (fig. 2) that the same pattern of excretion of radioactivity occurred.

Previous studies (10, 11) have demonstrated the existence in the rat of at least two pathways for thiamine degradation. One is the "cleavage" pathway in which the molecule is split into its two moieties and the other is the "ring opening" pathway in which the thiazole ring opens and the number 2 carbon is released as CO_2. If these two pathways are considered to compete for available thiamine, then a speculative explanation for these observations may be outlined. A low oxythiamine-to-thiamine ratio (2:1) increases the amount of $^{14}CO_2$ generated from thiamine but decreases the urinary excretion of thiamine and its metabolites (see table 1). Because oxythiamine at low levels of intake is not readily phosphorylated (12) it may be more susceptible to destruction by cleavage and thus competes favorably with thiamine for the enzyme(s) involved. Consequently, although some thiamine is spared catabolism by the cleavage pathway it is diverted to the ring-opening pathway for destruction. Oxythiamine is, in fact, more susceptible to cleavage than thiamine under these conditions (13).

When greater oxythiamine-to-thiamine ratios are used, the interpretation becomes more complex. When ratios of 4:1 or 6:1 are used it is possible that oxythiamine can now compete even more vigorously with thiamine in both the "cleaving" and "ring-opening" pathways. Accordingly, both the generation of $^{14}CO_2$ from thiamine and the excretion of thiamine-urinary metabolites are further reduced. It is also probable

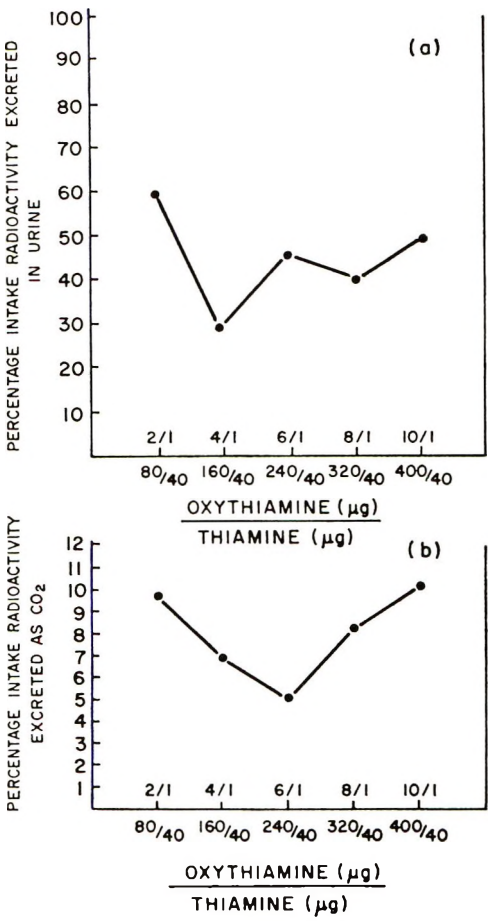

Fig. 2 Percentage of injected radioactivity appearing in urine and expired CO_2 in individual rats of group 2 given different ratios of oxythiamine to ^{14}C-thiamine. Each rat first received the regular 2:1 injection and CO_2 and urine were collected for the next 24 hours. Then, the same rat was injected with a different ratio of oxythiamine to ^{14}C-thiamine, and CO_2 and urine were collected for the next 24-hour period.

that oxythiamine-thiamine competition in the renal tubule may account for at least a portion of the change in urinary excretion.

The highest oxythiamine-to-thiamine ratio used (10:1) is a level sufficient to produce chronic thiamine deficiency in the rat although most investigators have used much higher doses (1–4). Under these circumstances it may be presumed that oxythiamine is sufficiently phosphorylated to compete with thiamine in its usual metabolic pathways. Accordingly, thiamine pyrophosphate displaced from transketolase, pyruvic dehydrogenase, and the like, may be catabolized at an enhanced rate both by "cleavage" and by "ring-opening." Again, possible oxythiamine-thiamine competition in the renal tubule cannot be ignored since it is known that increased thiamine excretion occurs under such conditions.

To examine for the catabolic products of oxythiamine and thiamine, 6-day urine pools from each group were processed for column chromatography as described. Concentrates of radioactive metabolites from charcoal containing 65%, 65% and 60% of the total activity in the 6-day urine pools of groups 1, 2 and 3, respectively, were chromatographed on Amberlite CG-50 and the patterns are seen in figure 3.

The radioactive metabolites of group 1 (^{14}C-thiamine alone) were resolved into five peaks whereas group 3 (^{14}C-oxythiamine + unlabeled thiamine) were resolved into two large peaks and two very small peaks (fig. 3).

Peaks I and III from the urine of ^{14}C-oxythiamine-treated rats were larger than those of ^{14}C-thiamine-injected rats. In addition, a small peak IV was also seen in the urine of ^{14}C-oxythiamine-treated rats. This peak is of particular interest since it normally contains thiamine. The radioactivity pattern of group 2 (^{14}C-thiamine + unlabeled oxythiamine) was similar to that of group 1 except that the peak containing thiamine was relatively greater and peak I was relatively smaller.

The contents of the tubes containing the principal radioactive peaks were combined and a small aliquot was taken from each peak for liquid-scintillation counting. The radioactivities of these peaks, as the percent of the total radioactivity applied to the Amberlite column are summarized in table 3. Most of the radioactivity from ^{14}C-oxythiamine appears in peak III (81%) and peak I (15%). There is, in addition, a small peak prior to peak I (fig. 3, peak 0) in both groups which is the result of spontaneous breakdown of ^{14}C-thiamine and ^{14}C-oxythiamine. Peak I from ^{14}C-thiamine-injected animals is larger than that from the rats injected with ^{14}C-thiamine + oxythiamine. The main component of peak IV is thiamine. The percentage of radioactivity in this peak was 25% in the ^{14}C-thia-

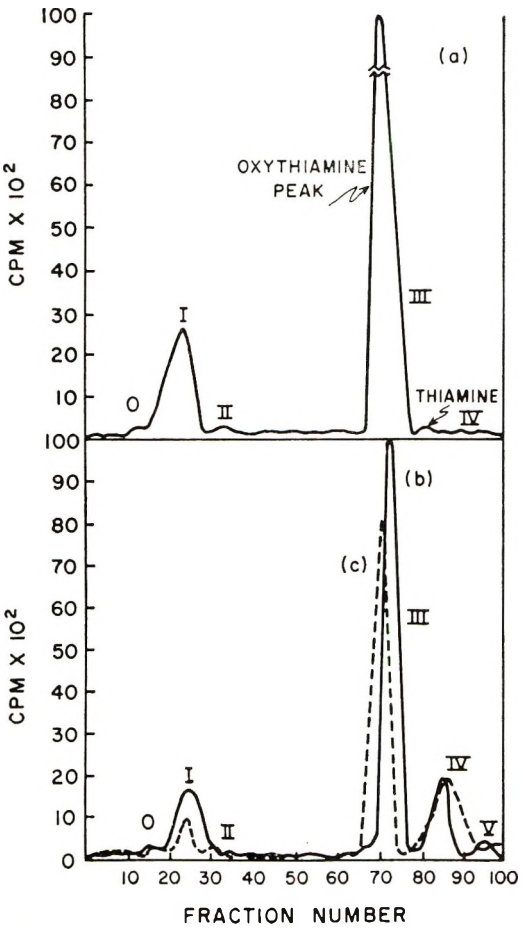

Fig. 3 A comparison of the radioactive elution patterns from Amberlite CG-50 chromatograms of urines from the three groups of rats. Exchanger, Amberlite CG-50 (200 to 400 mesh), 1 cm x 40 cm, in the H form. Flow rate 75 ml/hour, fraction size 5 ml. Nearly identical amounts of radioactivity were applied to each column. a) Elution pattern of ^{14}C-oxythiamine, b) elution pattern of ^{14}C-thiamine, and c) elution pattern of ^{14}C-thiamine + unlabeled oxythiamine (·– – – –·).

mine-injected rats (group 1) and 34% in the ^{14}C-thiamine + oxythiamine-injected rats (group 2). Thus, oxythiamine increases the excretion of thiamine in the urine when an oxythiamine-to-thiamine ratio insufficient to retard growth is used as well as when sufficient oxythiamine is used to induce a chronic deficiency (6).

Each radioactive peak was reduced in volume under vacuum in a rotary evaporator. The resulting concentrate was then lyophilized and the residue was taken up in a small volume of water. The concentrates obtained by this method occasionally contained some extraneous materials which were insoluble in the small volume of water. This material which contained little radioactivity, was normally removed by centrifuging (5000 × g) for 15 minutes. Identical peak concentrates from the three groups of rats were chromatographed on thin-layer cellulose plates. They were spotted side by side for development in a solvent consisting of n-propanol–acetate buffer–water. The resulting thin-layer chromatograms were exposed to X-ray films for preparation of autoradiograms. After development of the X-ray films, the radioactive metabolites were localized on the plates and the cellulose at the appropriate locale was removed with a microsuction device. For characterization studies, the metabolites were eluted from the cellulose with a small volume of water.

In general, fewer radioactive metabolites were found in the urine of the ^{14}C-oxythiamine treated rats than in the urine of the ^{14}C-thiamine-injected animals. Approximately 25 radioactive compounds were found in the urine of rats receiving ^{14}C-thiazole-labeled thiamine (excluding peak V), and 14 were found in the urine of the rats injected with ^{14}C-oxythiamine. Table 4 shows the R_F values in the major radioactive peaks.

The major component of peak I (R_F 0.80) is present in all three groups of rats, i.e., it is derived both from thiamine and oxythiamine. The isolation and identification of this compound as 4-methyl-5-thiazoleacetic acid is described in another publication (13). In addition, another metabolite in this peak (R_F 0.73) occurs only in rats injected with labeled or unlabeled oxythiamine. The nature of this compound is unknown.

Peak II from ^{14}C-thiamine-treated rats contained more radioactive metabolites than did the comparable peak from ^{14}C-oxythiamine-injected rats. Because this peak contains only a small portion of the total radioactivity in the urine further studies were not attempted.

A major portion of the administered ^{14}C-oxythiamine appeared intact in peak III (R_F 0.45). About 55% of the injected daily dose appeared as such with 24 hours of

TABLE 3

Distribution of radioactivity among the peaks obtained from Amberlite CG-50 chromatography of desalted urine from three groups of rats [1]

Peaks no.	Group 1, ^{14}C-thiamine	Group 2, ^{14}C-thiamine + oxythiamine	Group 3, ^{14}C-oxythiamine + thiamine
0	4.9	3.7	1.2
I	10.7	5.9	15.3
II	1.2	1.4	1.4
III	48.8	46.0	81.2
IV	25.4	34.4	0.50
V	2.4	2.9	—

[1] The values represent the metabolites from a pooled 6-day urine sample collected from 6 rats in each group and are expressed as the percentage of the total radioactivity applied to the Amberlite column.

TABLE 4

Average and range of R_F values of the radioactive metabolites of 2-^{14}C-thiazole-labeled oxythiamine found in rat urine [1]

Peak I	Peak III	Peak IV
1. 0.33–0.39(0.36) [2]	1. 0.41–0.48(0.45) [2,3]	1. 0.39–0.42(0.40) [2,3]
2. 0.40–0.46(0.43) [3]	2. 0.47–0.57(0.52) [3]	
3. 0.52–0.57(0.54)	3. 0.82–0.87(0.85) [3]	
4. 0.70–0.76(0.735)		
5. 0.79–0.83(0.80) [2,3]		

[1] The metabolites were isolated by subjecting the radioactivity concentrated from rat urine to ion exchange chromatography on Amberlite CG-50 followed by cellulose thin-layer chromatography of the concentrated peaks using the solvent system *n*-propanol – water – 1 M acetate buffer pH 5.0 (70:20:10, by volume).
[2] Major components are underscored.
[3] Indicates a metabolite apparently common to both ^{14}C-thiamine and ^{14}C-oxythiamine. The average R_F value is shown in parentheses.

injection. A narrow radioactive band was found in peak III of groups 1 and 2 which had an R_F value identical to that of oxythiamine in the *n*-propanol–acetate buffer–water solvent system. Further studies are required to determine if this compound is indeed oxythiamine. Two major unidentified metabolites of thiamine occur in peak III. Neither, however, have R_F values coinciding with the oxythiamine metabolites found in this peak.

In earlier studies, we found no evidence for the presence of free thiazole in rat urine. In the present study, however, traces of this metabolite were detected. Thiazole appeared in extremely small amounts nearly at the solvent front (R_F 0.85) in peak III autoradiograms of all three groups. A subjective impression was that more thiazole appeared in the urine of the rats injected with ^{14}C-oxythiamine but that total urinary radioactivity present as this compound was less than 1.0% in all three groups.

Chromatography of the pooled urines of ^{14}C-oxythiamine-injected rats yielded a small peak IV which normally contains thiamine. Cellulose thin-layer chromatography of this peak showed that ^{14}C-thiamine was indeed present (see table 4) suggesting either that oxythiamine was being converted by the rat to thiamine or that the ^{14}C-oxythiamine was contaminated with small quantities of ^{14}C-thiamine. Accordingly, a new batch of ^{14}C-oxythiamine was prepared (see Methods) purified by Amberlite CG-50 chromatography. Only a trace of ^{14}C-thiamine was detected by this procedure which cleanly separates oxythiamine from thiamine. The radioactive peak corresponding to purified oxythiamine was collected and evaporated under reduced pressure. Four, adult female rats of the Sprague-Dawley strain were maintained on a thiamine-free diet and injected daily for 14 days with 80 μg of the purified ^{14}C-oxythiamine + 40 μg unlabeled thiamine. The urine of these rats was

pooled and processed on Amberlite CG-50 as described previously. A small peak IV containing thiamine was again isolated but again in a very small amount.

Additional evidence for this conversion was then sought. The livers from rats injected with ^{14}C-oxythiamine were homogenized in 5% trichloroacetic acid and the protein precipitate was discarded. The supernatant extract was neutralized with sodium hydroxide and stirred with acid-washed charcoal. The radioactive metabolites were eluted from the charcoal with pyridine–ethanol–water and the concentrated eluate was then subjected to Amberlite CG-50 column chromatography. A very small thiamine peak was collected. Thin-layer chromatography of this preparation confirmed the existence of a small amount of radioactive thiamine.

The possibility that the thiamine detected was a product of the bacterial flora of the gut was then examined. The collected feces of the rats injected with purified ^{14}C-oxythiamine were extracted by steaming in 0.2 N HCl. The resulting extract was centrifuged to remove the precipitate and the precipitate was reextracted with another volume of 0.2 N HCl. The supernatant solutions were combined and the pH was adjusted to 6.5 with NaOH. It was then subjected to the same charcoal-absorption precedure used with urine with subsequent elution of the radioactivity with pyridine–ethanol–water. The latter was concentrated and chromatographed on a column of Amberlite CG-50. A pooled-feces collection (19 days) obtained from group I which received ^{14}C-thiamine alone was processed simultaneously.

The patterns of radioactivity obtained for both groups are shown in figure 4. There is a "notch" in peak III of the feces extract from the ^{14}C-oxythiamine-treated rats. Because it was not clear whether this represented a counting artifact or overlapping peaks, each part of the peak was collected separately, its volume reduced and lyophilized, and the concentrates were chromatographed on cellulose thin-layer plates using the *n*-propanol–acetate buffer –water solvent system. Both portions of the peak were found to contain a single radioactive compound with an R_F value identical to that of ^{14}C-oxythiamine. These compounds were eluted from the cellulose thin-layer plates, combined with an authentic sample of ^{14}C-oxythiamine and rechromatographed on an Amberlite CG-50 column. A single symmetrical peak appeared in the position usually assumed by peak III — no thiamine was detected. A relatively large thiamine peak, however, was found in the feces of the ^{14}C-thiamine-injected rats. This is in contrast to the findings of Pearson et al. (14), who did not detect any radioactive thiamine in the feces of rats injected with ^{14}C-pyrimidine-labeled thiamine. We can offer no expla-

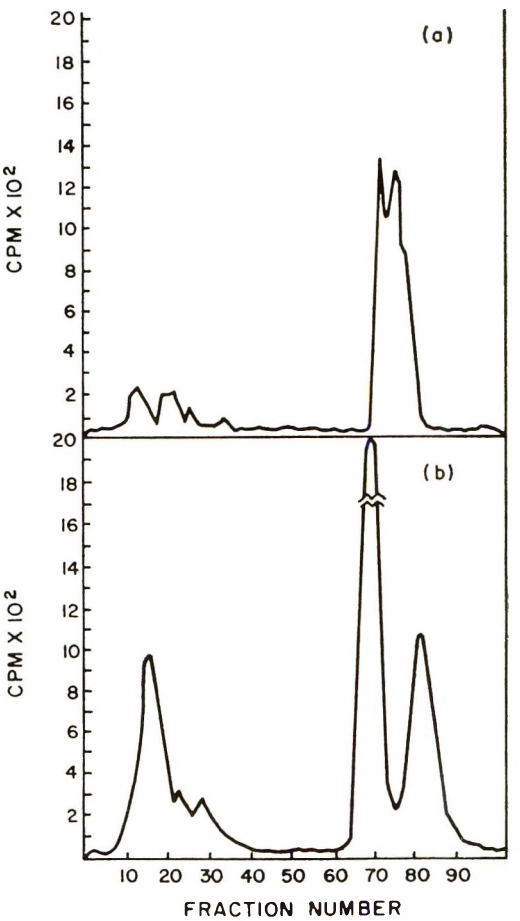

Fig. 4 Comparison of the chromatographic behavior of the fecal metabolites of a) ^{14}C-oxythiamine and b) ^{14}C-thiamine. The resin was Amberlite CG-50 (200 to 400 mesh), 1.0 x 40.0 cm, in the H form. Flow rate 60 ml/hour, fraction size 5 ml.

nation for this anomaly other than that the extraction procedures differed and different species of ^{14}C-thiamine were used.

The failure to detect even a small amount of ^{14}C-thiamine in the feces extract of ^{14}C-oxythiamine-injected rats would seem to exclude the possibility that the ^{14}C-thiamine found in the ^{14}C-oxythiamine-treated rats was of bacterial origin. We consider, accordingly, that rat tissues possess a limited capability to convert oxythiamine to thiamine. The quantity converted, however, is so small that no benefit accrues to the animal. There is a precedent for this finding inasmuch as the conversion of oxythiamine to thiamine through formation of an intermediate "thiaminosuccinic acid" by a thiamineless mutant of *Escherichia coli* has been reported by Fukui et al. (15).

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Oxidation of Exogenous Reduced Nicotinamide Adenine Dinucleotide by Liver Mitochondria from Essential Fatty Acid-deficient Rats [1]

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ABSTRACT The oxidation of exogenous NADH by liver mitochondria from normal rats and rats reared with diets deficient in the essential fatty acids was studied. When an isotonic medium was employed, the deficient mitochondria carried out the oxidation at measurable rates, whereas normal ones did not. Under hypotonic conditions the deficient mitochondria oxidized NADH at much slower rates than did normal mitochondria. Following disruption of the mitochondria by sonication, the two preparations carried out the oxidation at similar rates. Starvation of normal rats followed by a short-term feeding of the essential fatty acid-deficient diets gave rise to lowered rates of NADH oxidation by the mitochondria in the hypotonic reaction medium. When essential fatty acid-sufficient diets were re-fed, NADH oxidation rates appeared approximately normal. The lowered rate of NADH oxidation by liver mitochondria in the hypotonic reaction medium appeared to be a characteristic of an essential fatty acid deficiency, and can be a simple biochemical criterion of it. It was concluded that the deficiency affected the permeability of the liver mitochondria to NADH.

It has been known for many years that exogenous NADH is not oxidized by intact mitochondria. The oxidation proceeds readily, however, if the mitochondria are disrupted or are merely suspended in a hypotonic medium (1). The latter presumably alters membrane permeability, allowing NADH access to sites of respiratory enzymes within the mitochondria.

Previous studies (2, 3) showed that an essential fatty acid deficiency in rats results in liver mitochondria which readily lose oxidative phosphorylation, ATP-Pi exchange activity, and respiratory control. These observations led to the suggestion that in an essential fatty acid deficiency, liver mitochondria are structurally more labile, and membrane permeability probably is altered. In the present investigation, the nature of this mitochondrial lability with possible membrane alteration is studied further by examining rates of oxidation of NADH by normal and deficient rat liver mitochondria under varying conditions of medium tonicity, or by sonically disrupted mitochondria.

EXPERIMENTAL PROCEDURE

The experimental animals were rendered essential fatty acid-deficient as described previously (3). They were divided into two groups at weaning; one group was fed the fat-free diet, with the second group receiving the diet in which hydrogenated coconut oil or tripalmitin replaced sucrose at a level of 5%. The normal control rats were in two groups, one group receiving from weaning the fat-free diet in which corn oil replaced sucrose at a level of 5%, the other group receiving a commercial pelleted diet. All animals were maintained with the diets indicated 12 to 16 weeks before they were killed.

The hypotonic reaction medium consisted of 0.015 M potassium phosphate buffer, pH 7.4, 0.005 M $MgCl_2$, 0.005 M KCl and 0.0001 M EDTA. The isotonic reaction medium contained 0.25 M D-mannitol in addition to the above ingredients. Mitochondria were disrupted by the use of ultrasonic oscillation for two minutes.[3] The disrupted mitochondria were used without fractionating. Oxygen uptake was determined following the addition of 0.2 ml of

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[3] Branson Instruments sonifier model LS-76, 20 kc, power setting 6.

suspended mitochondria to 2.8 ml of one of these media. To each reaction vessel (final volume 3 ml) was added 50 µl of a solution containing 20 mg NADH [4]/ml in 0.1 M Tris buffer, pH 7.4. NADH oxidation was determined polarographically using an oxygen electrode (3). Mitochondrial protein was determined by the Folin phenol reagent modified by Miller (4).

RESULTS

Effects of tonicity on NADH oxidation by intact mitochondria. The intact mitochondria were added to reaction mixtures of different tonicities (containing different amounts of D-mannitol) and three minutes later NADH was added. The rates of NADH oxidation were calculated from the slopes obtained on the polarograph record, by subtracting the rate of oxygen uptake in the absence of NADH from that in its presence. When conditions of near-isotonicity were used (that is, with concentrations of D-mannitol in excess of 0.1 M) the normal mitochondria failed to oxidize NADH to any appreciable extent (fig. 1). The deficient mitochondria, however, oxidized NADH at low but measurable rates. At D-mannitol concentrations lower than 0.1 M, the rate of NADH oxidation by the normal mitochondria increased rapidly with decreasing D-mannitol concentrations (that is, with increasing hypotonicity). In view of the relative abilities of normal and deficient mitochondria to oxidize NADH in D-mannitol concentrations above 0.1 M, it was unexpected to find that deficient mitochondria oxidized this substrate relatively slowly at D-mannitol concentrations below 0.1 M (fig. 1).

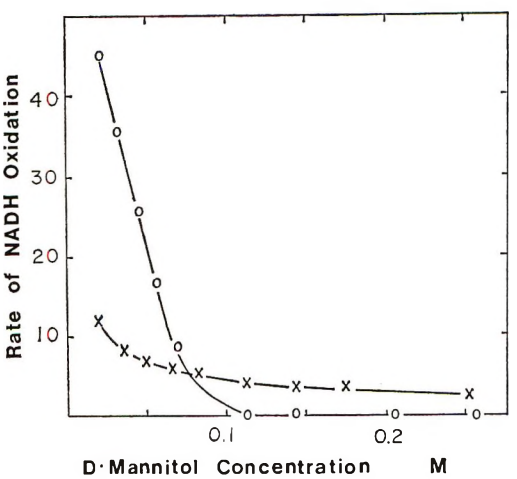

Fig. 1 Effect of D-mannitol on rate of NADH oxidation of intact liver mitochondria of normal and fat-deficient rats. O, normal (5% corn oil) diet; X, deficient (fat-free) diet. Ordinate, number of µmoles oxygen consumed per minute per milligram protein per milliliter reaction mixture; and abscissa, molar concentration of mannitol in the reaction mixture.

Results similar to those described above were obtained when D-mannitol was replaced by sucrose.

Dietary effects on NADH oxidation. In the course of the above studies it was observed that the rate of NADH oxidation, calculated on a mitochondrial protein basis, occasionally varied considerably even within a group of animals fed the same diet, but that it appeared to be roughly proportional to the rate of State 3 (5) succinate oxidation. Therefore, the rate of NADH oxidation was compared with rate of succinate oxidation at State 3, and the ratio NADH oxidation rate to State 3 succinate oxidation rate was defined as the "specific activity" of NADH oxidase. This ratio represents the rate of NADH oxidation of mitochondria relative to respiratory chain enzyme activity and has the advantage that it is not affected by the possible presence, in individual mitochondrial preparations, of varying amounts of proteins other than respiratory chain enzymes.

When reactions were carried out in an isotonic medium, liver mitochondria from the rats fed the diets deficient in essential fatty acids consistently demonstrated higher NADH oxidase activity than did mitochondria from the rats fed 5% corn oil diet or commercial pelleted ration. Under conditions of hypotonicity, the reverse was true (table 1). When, however, the mitochondria were disrupted by sonication, similar rates of oxidation of NADH in the normal and deficient animals were observed. This was due to a three- to four-fold increase in NADH oxidation by deficient mitochondria, and no change in oxidative activity in normal ones, following sonication (table 1).

The question arose whether the decreased NADH oxidase activity measured

[4] Sigma Chemical Company, St. Louis.

in the hypotonic medium might be associated with the conditions described by Allman et al. (6), who observed marked changes in liver lipids in normal rats fed a diet deficient in fats for a short period of time after starvation. Use was made of their observation in the following way.

Healthy male adult rats were starved for two days and re-fed diets of different fat composition for a period of one week. They were then killed and NADH oxidase activity was determined in mitochondrial preparations as described above. There were decreases in the NADH oxidation rates in the hypotonic reaction medium by liver mitochondria of rats re-fed either the fat-free diet, the 5% hydrogenated coconut oil diet, or the 5% tripalmitin diet for one week. Sonicated liver mitochondria from the rats fed the three essential fatty acid-deficient diets had as high NADH oxidase activity as the sonicated mitochondria prepared from the rats fed the two normal diets (table 2).

TABLE 1

NADH oxidation by liver mitochondria of normal and essential fatty acid-deficient rats [1]

Diets	Specific activity of NADH oxidase		
	Intact mitochondria		Sonicated mitochondria
	Iso- [2]	Hypo- [2]	Hypo- [2]
Commercial pelleted diet	0.012 [3] (0.000–0.020)	0.520 (0.492–0.545)	0.609 (0.572–0.622)
Corn oil	0.007 (0.000–0.012)	0.652 (0.625–0.680)	0.628 (0.596–0.642)
Fat free	0.039 (0.015–0.048)	0.215 (0.196–0.230)	0.775 (0.722–0.812)
Hydrogenated coconut oil	0.032 (0.020–0.045)	0.185 (0.160–0.194)	0.655 (0.584–0.720)

[1] See experimental procedure for the diets indicated. Specific activity is defined in the text.
[2] The columns designated as iso- and hypo- represent the rates measured in isotonic and in hypotonic reaction media, respectively.
[3] The activities indicated are the mean of 5 rats. Figures in parentheses are ranges of values.

TABLE 2

NADH oxidation by liver mitochondria from healthy adult rats fed various diets for one week after 48 hours of starvation [1]

	Specific activity of NADH oxidase		
	Intact mitochondria		Sonicated mitochondria
	Iso- [2]	Hypo- [2]	Hypo- [2]
Commercial pelleted diet	0.013 [3] (0.004–0.023)	0.473 (0.448–0.495)	0.591 (0.555–0.636)
Corn oil	0.020 (0.007–0.028)	0.415 (0.392–0.445)	0.552 (0.504–0.588)
Hydrogenated coconut oil	0.019 (0.010–0.026)	0.251 (0.230–0.272)	0.702 (0.625–0.730)
Tripalmitin	0.015 (0.007–0.022)	0.221 (0.185–0.238)	0.627 (0.580–0.678)
Fat free	0.025 (0.013–0.034)	0.258 (0.216–0.270)	0.586 (0.525–0.612)
Fasting only	0.017 (0.008–0.026)	0.333 (0.305–0.354)	0.545 (0.488–0.572)

[1] See experimental procedure for the diets indicated. Specific activity is defined in the text.
[2] The columns designated as iso- and hypo- represent the rates measured in isotonic and hypotonic reaction media, respectively.
[3] The activities indicated are the mean of 4 rats except the case of "fasting only" in which the mean values of 6 rats were shown. Figures in parentheses are ranges of values.

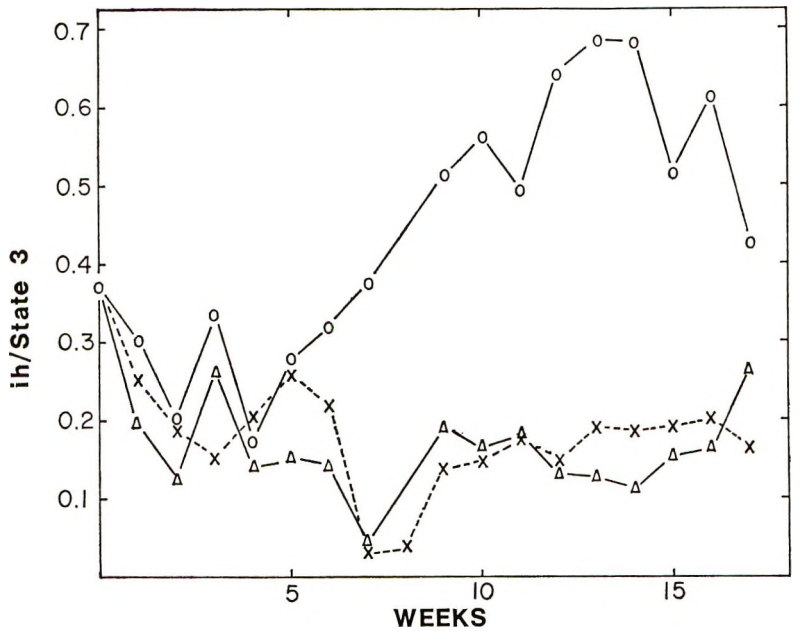

Fig. 2 NADH oxidation by liver mitochondria of rats during the development of essential fatty acid deficiency. O, normal (5% corn oil) diet; X, fat-free diet; and △, hydrogenated coconut oil (5%) diet. Ordinate, ih/State 3, specific activity of NADH oxidation (defined in the text) of intact mitochondria in the hypotonic reaction medium; and abscissa, weeks after the rats were placed on the different diets.

NADH oxidation by liver mitochondria of rats during the development of essential fatty acid deficiency. Weanling rats were fed either the control diet containing 5% corn oil, the fat-free diet, or the diet containing 5% hydrogenated coconut oil. NADH oxidation by the liver mitochondria prepared from those rats was determined weekly. The NADH oxidase activity of the intact mitochondria in the hypotonic reaction medium fluctuated considerably in all the groups of rats for the first five or six weeks when the rats were in the early period of rapid growth. Thereafter it increased steadily in the rats fed the corn oil diet and decreased or failed to change in the rats fed either fat-free or 5% hydrogenated coconut oil diets (fig. 2).

DISCUSSION

The low rate of NADH oxidation by liver mitochondria prepared from essential fatty acid-deficient rats appears, from these studies, to be a biochemical expression of the deficiency, and may be used as a simple biochemical criterion for it. It cannot be assumed, however, to be specific for the deficiency.

An important question posed by the data of table 1 and figure 2 is whether an alteration of the composition of liver fatty acids that occurs in the classical essential fatty acid deficiency is responsible for the lowered NADH oxidase specific activity observed here. It cannot be assumed that starving the rats, followed by a short period of refeeding with an unsaturated fatty acid-deficient diet, results in the same physiological circumstances seen in rats fed an essential fatty acid-deficient diet over a longer period of time. It is clear, however, that this procedure results in a lowered NADH oxidase specific activity, similar to that seen in a classical deficiency (compare data in tables 1 and 2). The data presented here, together with those of Allman et al. (6), suggest that the biochemical end-point employed in the present experiments is indeed associated with the altered fatty acid composition of liver mitochondria that occurs in the classical essential fatty acid deficiency (7). Fur-

ther work is necessary, however, to clarify and substantiate this relationship.

Despite the fact that under hypotonic conditions, the deficient mitochondria carry out the oxidation of NADH relatively poorly, they apparently are not deficient in any of the respiratory chain components. This is concluded since sonic disruption of the mitochondria resulted in an NADH oxidase activity in the deficient mitochondria equal to that seen in the normal ones. Thus, we have the following interesting set of circumstances. When an isotonic medium is used, deficient mitochondria can carry out the oxidation of NADH at measurable rates, whereas normal ones cannot. Hypotonic conditions apparently render the membrane of the latter more permeable and allow the oxidation to proceed at much faster rates than those seen with deficient mitochondria. Inasmuch as sonic disruption did not further increase the oxidation rates, hypotonicity apparently rendered exogenous NADH accessible to respiratory chain components, and sonic disruption made it fully accessible.

These observations provide a further indication that a defect exists in the membrane of the liver mitochondria of the essential fatty acid-deficient rat. It is suggested that for a normal mitochondrial membrane to be permeable to NADH under hypotonic conditions, certain steric factors are required. These appear to be dependent on the presence of essential fatty acids. This requirement may not be met in the membrane composed of structural proteins and phospholipids containing small amounts of the essential fatty acids. Thus, the low rate of NADH oxidation takes place despite the increased swelling tendency and the enhanced membrane lability.

Inasmuch as the alteration appears to be in the area of the complex I enzymes in the respiratory chain described by Hatefi et al. (8), it would seem worthwhile to consider this a possible site of a biochemical aberration in an essential fatty acid deficiency.

ACKNOWLEDGMENT

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Effect of Protein Intake on Tissue Amino Acid Levels and the Enzymes of Serine Biosynthesis in the Rat [1]

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ABSTRACT Enzymes of serine metabolism including 3-P-glycerate dehydrogenase, P-serine phosphatase, D-glycerate dehydrogenase and serine dehydratase were measured in several tissues of the rat under varying dietary conditions. The enzymes of the phosphorylated pathway of serine biosynthesis were present in liver, kidney and brain, but not in heart, muscle or intestine. Low protein diets resulted in marked increases in the activity of 3-P-glycerate dehydrogenase and P-serine phosphatase in liver, but not in kidney or brain. This increased enzyme activity in liver was correlated with a depression in the tissue concentration of cystine, methionine and taurine. Supplementation of the diet with methionine or cystine reversed the enzyme changes and partially reversed the decrease in sulfur-containing amino acid levels in the liver. No changes in amino acid content occurred in kidney and brain with the diets or supplements studied. The data suggest the probable association of the concentration of specific amino acids with levels of 3-P-glycerate dehydrogenase in various tissues. A depression of hepatic serine, glycine, and alanine occurred when the low protein diet was supplemented with methionine. Methionine completely prevented the rise of 3-P-glycerate dehydrogenase in the protein-depleted rats. This observation demonstrates the importance of the phosphorylated pathway of serine biosynthesis in maintaining hepatic serine biosynthesis in rats fed low protein diets.

Two potential pathways of serine biosynthesis from glycolytic intermediates are described in mammalian tissues (1, 2) (fig. 1). Enzymes of both pathways are present in rat liver, but previous studies have demonstrated that alterations in enzyme activity in response to dietary protein depletion were limited to the "phosphorylated pathway" (3). 3-P-Glycerate dehydrogenase and P-serine phosphatase are increased by feeding protein-deficient diets (3, 4). It is likely that these changes are mediated by an increase in enzyme synthesis (3, 5). The increase in enzyme activity is prevented by addition of 1% cysteine to the diet (3), but the mechanism of this effect has not been clarified.

The present studies describe the distribution of enzymes of serine metabolism in various rat tissues and the activity of these enzymes in rats fed protein-deficient diets. Enzyme activity was correlated with the corresponding tissue free amino acid levels when rats were fed diets containing 25% casein, 2% casein or 2% casein supplemented with cysteine and methionine. Low protein diets resulted in changes in free amino acid levels only in the liver. Corresponding changes in 3-P-glycerate dehydrogenase, P-serine phosphatase and serine dehydratase were also limited to the liver. The data suggest a possible correlation between tissue amino acid concentration and the level of the enzymes of serine biosynthesis.

The physiological importance of the phosphorylated pathway in the response of the organism to protein starvation is suggested by the decline in tissue serine and glycine levels when compensatory changes in this pathway were prevented by dietary-methionine supplementation.

METHODS

Male rats of the Sprague-Dawley strain were used for all studies. The 2% or 25% casein diets were prepared as described previously (3). Cysteine, methionine, or other amino acids were added to the 2% casein diet in the concentration of 2 g/100 g diet. The rats were pair-fed each of the special diets for 7 days and killed without fasting. The preparation and dialysis of enzyme fractions from brain, kidney, small intestine, heart and muscle

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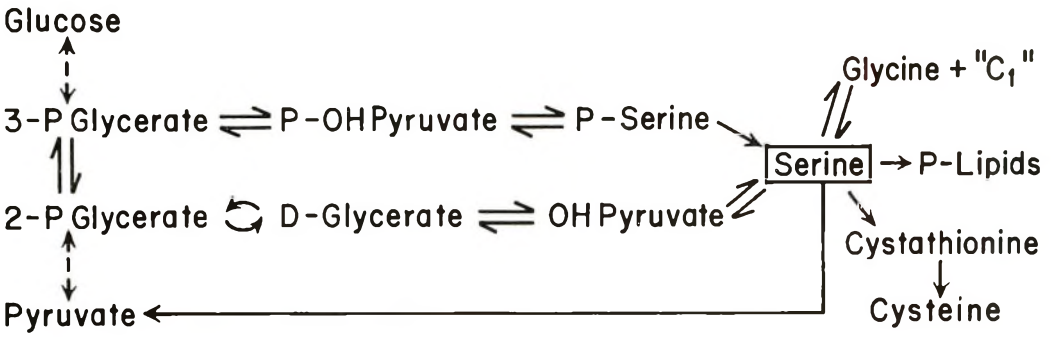

Fig. 1 The pathways of serine biosynthesis in rat liver. The upper, or "phosphorylated" pathway includes 3-P-glycerate dehydrogenase, a transaminase and P-serine phosphatase. The "nonphosphorylated" pathway is below. Serine is converted to pyruvate by serine dehydratase. "C_1" refers to methyl groups (N5, N^{10}-methylene tetrahydrofolate).

were as previously outlined for liver (3). The assays for 3-P-glycerate dehydrogenase, P-serine phosphatase, D-glycerate dehydrogenase and serine dehydratase have been previously described (3). Units were defined as μmoles of substrate converted per minute at 37°. All materials were obtained from described sources (3). Details or variations in methodology are found in figure and table legends.

Extracts for determination of free amino acids were prepared by boiling 1 g of wet tissue in 4 ml of water. The tissue was homogenized and 1 ml of 20% (w/w) sulfosalicylic acid added to 2 ml of homogenate to complete the precipitation of proteins. An aliquot of the supernate was analyzed for amino acids.

Amino acids were measured by an automatic amino acid analyzer [2] using a 120 cm × 0.9 cm column with Chromobead, type B resin,[3] and a 21.5-hour elution time. The nine-chamber Autograd device [4] was used for producing a smooth pH gradient between 2.875 and 4.3 and a continuous sodium gradient between 0.2 and 0.8 M. Composition of buffers were as described by Hamilton.[5]

RESULTS

The rats fed the 25% casein diet gained an average of 30 g during the 7-day feeding period. The rats fed the low protein diet, however, with or without amino acid supplements lost 15 ± 4% of their body weight.

Distribution of enzyme activity. 3-P-Glycerate dehydrogenase was measured in liver, kidney, brain and intestine, but no activity was demonstrated in skeletal muscle or heart. The linearity of 3-P-glycerate dehydrogenase activity with respect to time and enzyme concentration (fig. 2) was established for brain and kidney.

The Michaelis constant, K_m, for 3-P-glycerate was determined graphically with enzyme preparations from liver, kidney and brain (table 1). No substantial differences were observed in these studies.

Inhibition of bacterial 3-P-glycerate dehydrogenase by serine has been described (6), but serine, glycine, cysteine or combinations of these amino acids did not inhibit 3-P-glycerate dehydrogenase activity in liver, kidney or brain preparations.

P-serine phosphatase activity was demonstrated in brain and kidney but not in muscle, heart or intestine. Inhibition of P-serine phosphatase activity by 0.01 M L-serine was observed in brain and kidney, as previously demonstrated in liver (3, 7).

D-Glycerate dehydrogenase activity could be demonstrated in kidney and liver, but preparations of muscle, intestine and brain were inactive by the methods used. In contrast to the results of Walsh and Sallach (1), the specific activities of D-glycerate dehydrogenase and 3-P-glycerate dehydrogenase were equal in the kidney of rats fed normal diets. Serine dehydratase activity was limited to liver, and was markedly de-

[2] Technicon Instruments Corporation, Chauncey, New York.
[3] See footnote 2.
[4] See footnote 2.
[5] Hamilton, P. B. 1963 Ion exchange chromatography of amino acids. Anal. Chem., 35: 2055 (abstract).

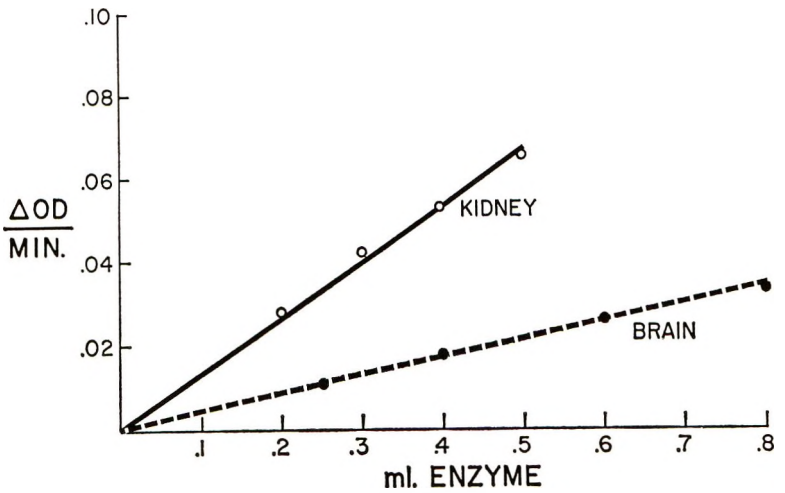

Fig 2 The relationship of enzyme concentration to activity is shown for 3-P-glycerate dehydrogenase. The assay was performed as described under Methods. A similar linear relationship was previously noted in liver preparations (3).

TABLE 1

Michaelis constants for 3-P-glycerate dehydrogenase

Tissue	K_m for 3-P-glycerate [1]
Liver	$2.78 \text{ M} \times 10^{-4}$
Brain	$1.28 \text{ M} \times 10^{-4}$
Kidney	$1.78 \text{ M} \times 10^{-4}$

[1] The K_m values were determined graphically. The substrates used were 3-P-glycerate and NAD. The NAD concentration was 0.65 mM.

pressed by low protein diets (table 2) as previously noted (3).

Effects of dietary protein on tissue enzyme activity. The activities of 3-P-glycerate dehydrogenase, P-serine phosphatase and serine dehydratase in liver were markedly altered by changes in dietary protein content (3). These changes are shown in table 2. In contrast to previous studies (3), the increase in 3-P-glycerate dehydrogenase associated with the 2% casein diet was not completely prevented by the addition of 2% cysteine. Methionine, however, completely prevented the increase in this enzyme activity as shown in table 3. The addition of taurine, glutamate, tryptophan, glycine, serine or sulfur was without effect on 3-P-glycerate dehydrogenase activity.

No dietary-induced alterations in 3-P-glycerate dehydrogenase, P-serine phosphatase or serine dehydratase occurred in the kidney or brain of these same animals, as shown in table 4. The activity of D-glycerate dehydrogenase in kidney was unal-

TABLE 2

Enzyme levels in liver [1,2]

Diet [3]	3-P-glycerate → P-hydroxypyruvate	P-hydroxypyruvate → 3-P-glycerate	P-serine phosphatase	Serine dehydratase
	units/g protein	*units/g protein*	*units/g protein*	*units/g protein*
25% casein	0.51 ±0.01	1.23 ±0.16	16.2 ±0.4	200 ± 52
2% casein	6.95 [4] ± 1.06	17.08 [4] ± 2.86	32.9 [4] ± 1.0	nm [6]
2% casein + 2% cysteine	2.29 [4] ± 1.16	4.49 [4] ± 2.01	27.9 [5] ± 1.2	nm [6]

[1] Values represent means of 6 rats ± SD.
[2] Rats weighed 150 to 250 g.
[3] Diets were fed for 7 days before death.
[4] Value different from appropriate control ($P < 0.01$).
[5] Value not different from 2% casein group, but significantly different from 25% casein group ($P < 0.01$).
[6] nm = not measurable.

TABLE 3

3-P-Glycerate dehydrogenase activity in liver [1]

Diet [2]	No. of rats	3-P-glycerate → 3-P-hydroxypyruvate
		units/g protein
25% casein	11	0.66 ± 0.10 [3]
2% casein	13	4.44 ± 0.93
2% casein + 2.5% methionine	13	1.00 ± 0.42
2% casein + 2% serine	10	4.70 ± 0.67
2% casein + 2% glycine	10	4.60 ± 0.31
2% casein + 2% tryptophan	3	4.11 ± 0.69
2% casein + 2.5% glutamic acid	12	3.61 ± 0.34
2% casein + 1.8% sulfur	10	4.58 ± 0.89
2% casein + 2.5% taurine	10	4.61 ± 0.63

[1] Data compiled from several individual experiments.
[2] Rats fed diet for 7 days before death.
[3] Values are means ± SD.

tered by diet, a result similar to that previously described for liver (3).

Effects of dietary protein on tissue amino acid levels. The distinctive effects of dietary cysteine and methionine on the enzymes of hepatic serine biosynthesis suggested a possible correlation of certain tissue amino acids with enzyme levels. Therefore, the content of amino acids in liver, brain and kidney was measured following various dietary changes. Table 5 shows the amino acid concentrations in the liver of rats fed 25% casein, 2% casein, and 2% casein diets supplemented with 2% cysteine. All major amino acid components are included. The concentrations of other amino acids were not altered by diet.

The 2% casein diet resulted in a significant decrease in the hepatic concentration of glutamic acid, cystine, taurine and methionine. Supplementation of this diet with cysteine partially or completely prevented the decline in glutamic acid, cystine and taurine. Methionine levels remained low. There was a tendency for serine, glycine, and alanine to decline in the rats fed 2% casein supplemented with 2% cysteine, although these changes were not statistically significant.

The concentration of amino acids in kidney and brain was measured in the same animals (table 6). No changes from the control were recorded in these tissues when rats were fed 2% casein or 2% casein plus 2% cysteine. Thus, changes in tissue amino acid content were limited to the liver, the only organ in which enzyme levels were altered by diet.

The changes in hepatic amino acid levels were studied further by supplementing the 2% casein diet with individual amino acids. The addition of 2% glutamic acid, taurine, glycine, serine and threonine was without effect on hepatic enzyme levels.

TABLE 4

Enzyme levels in kidney and brain [1]

Diet	3-P-glycerate → P-hydroxypyruvate	P-serine phosphatase	Serine dehydratase
	units/g protein	*units/g protein*	*units/g protein*
	Kidney		
25% casein	5.48 ± 0.12 [2,3]	13.2 ± 2.1 [3]	42.5 [3]
2% casein	5.93 ± 1.37	13.7 ± 1.9	37.7
2% casein + 2% cysteine	5.72 ± 0.62	14.2 ± 2.1	36.5
	Brain		
25% casein	4.25 ± 0.72	9.5 ± 1.9	12.4
2% casein	4.08 ± 0.96	9.3 ± 2.1	12.3
2% casein + 2% cysteine	4.46 ± 0.96	9.8 ± 2.7	12.1

[1] Tissue was obtained from the same rats shown in table 2.
[2] Values are means of 6 rats ± SD.
[3] Differences are not stastically significant.

TABLE 5

Comparison of free amino acids of liver in rats fed diets of 25% casein, 2% casein and 2% casein plus 2% cysteine

Amino acid [1,2]	Control, 25% casein (8) [3]	Deficient, 2% casein (8) [3]	Supplemented, 2% casein + 2% cysteine (6) [3]
	μmoles/g	*μmoles/g*	*μmoles/g*
Serine	4.79 ± 2.60 [4]	3.95 ± 1.91 [4]	3.75 ± 0.90 [4]
Threonine	3.57 ± 1.35	2.82 ± 1.28	3.23 ± 0.57
Glycine	3.68 ± 1.14	3.85 ± 0.65	3.40 ± 0.33
Alanine	5.68 ± 2.13	6.85 ± 1.61	3.06 ± 1.43 [6]
Glutamic acid	7.34 ± 3.23	3.67 ± 1.07 [6]	9.37 ± 3.24 [6,7]
Cystine [5]	0.96 ± 0.19	0.22 ± 0.07 [6]	0.32 ± 0.01 [6,7]
Taurine	5.81 ± 3.35	0.96 ± 0.69 [6]	8.38 ± 5.05 [6,7]
Methionine	0.20 ± 0.08	0.06 ± 0.02 [6]	0.07 ± 0.02 [6]

[1] The amino acids listed represent 75% of the compounds detectable by the ninhydrin reaction.
[2] The other amino acids measured, including leucine, isoleucine, valine, lysine, ornithine, tyrosine, phenylalanine and histidine, were not altered in rats fed 2% casein diets with or without the cysteine supplement.
[3] Figures in parentheses refer to numbers of rats. Rats weighed 150 to 250 g.
[4] Values recorded are means ± SD.
[5] The values for cystine include all the oxidized cysteine present in the sample.
[6] Different from control rats, $P < 0.05$.
[7] Different from 2% casein, protein-deficient rats, $P < 0.05$.

TABLE 6

Serine, taurine and cysteine levels in kidney and brain

Tissue [1]	Amino acid [2]	Diet	
		25% casein	2% casein [3]
		μmoles/g liver	*μmoles/g liver*
Kidney	Serine	0.82 ± 0.20 [4]	0.67 ± 0.58 [4]
	Taurine	0.73 ± 0.24	0.61 ± 0.18
	Cystine	0.10 ± 0.02	0.07 ± 0.04
Brain	Serine	0.96 ± 0.24	0.83 ± 0.58
	Taurine	1.79 ± 0.14	0.79 ± 0.28 [5]
	Cystine	0.09 ± 0.10	0.08 ± 0.06

[1] Rats were the same as those recorded in table 5.
[2] None of the other amino acids measured, including those recorded in table 5, were altered by feeding 2% casein.
[3] Similar results were observed when 2% cysteine was added to the 2% casein diet.
[4] Values recorded are means ± SD.
[5] Group contained 3 rats.

Inorganic sulfur was also without effect. However, 2% methionine prevented the increase in enzyme levels and also altered tissue amino acid levels.

Table 7 shows the effects of methionine supplementation on liver amino acid concentration. Methionine and taurine were increased and serine, glycine and alanine were significantly decreased by dietary-methionine supplements. No change was noted in cystine content. Thus, supplementation of the protein-deficient diet with either cysteine or methionine will reverse changes in hepatic enzyme levels and result in similar alterations in liver amino acid concentrations.

Taurine levels were increased by cysteine, taurine and methionine supplements, but 2% taurine supplements were ineffective in preventing increases in enzyme activity. Supplementation of the 2% casein diet with taurine was unassociated with any of the amino acid changes noted when methionine and cysteine were added to the diet. The change in taurine levels in response to dietary methionine and cysteine is compatible with prior observations on the pathway of taurine synthesis (8, 9).

DISCUSSION

Enzymes of the "phosphorylated pathway" of serine biosynthesis are present in

TABLE 7

Liver amino acid concentration in rats fed 2% casein and 2% casein supplemented with 2% methionine

Amino acid	Diet [1]	
	Deficient, 2% casein	Supplemented, 2% casein + 2% methionine
	μmoles/g	*μmoles/g*
Serine	1.73 ± 0.21	0.77 ± 0.45 [2]
Threonine	1.16 ± 0.17	1.71 ± 0.75
Glycine	1.96 ± 0.39	0.55 ± 0.17 [2]
Alanine	3.28 ± 0.69	1.32 ± 0.26 [2]
Glutamic acid	2.27 ± 0.89	2.07 ± 0.49
Cystine	0.22 ± 0.03	0.18 ± 0.05
Taurine	0.59 ± 0.29	5.29 ± 2.47 [2]
Methionine	0.04 ± 0.02	0.36 ± 0.28 [2]

[1] Values are means ± SD for 6 rats in each group. Rats weighed 80 to 100 g.
[2] Difference significant, $P < 0.01$.

the liver, kidney and brain of rats. D-Glycerate dehydrogenase, an enzyme of the "nonphosphorylated pathway" is present only in kidney and liver. Therefore, only liver and kidney have the potential for serine biosynthesis by both pathways.

The levels of 3-P-glycerate dehydrogenase in kidney and brain of rats fed normal diets are 10-fold higher than in liver. The low level of 3-P-glycerate dehydrogenase in adult rat liver suggests this enzyme may be rate-limiting in the "phosphorylated pathway." This is supported by studies of Pizer (10) demonstrating absence of serine formation from 3-P-glycerate-^{14}C in liver extracts from adult rats fed normal diets. The high level of 3-P-glycerate dehydrogenase activity in brain may explain the observed synthesis of serine from carbohydrate precursors in this tissue (11).

In contrast to liver, the 3-P-glycerate dehydrogenase in kidney and brain does not show the marked increase in activity associated with low protein diets. This difference might be explained if separate enzyme proteins catalyzed this reaction in various tissues. Purification of 3-P-glycerate dehydrogenase from individual organs has not been achieved, but kinetic studies of several crude tissue preparations do not show significant differences.

The marked sensitivity of hepatic 3-P-glycerate dehydrogenase levels to dietary supplements of cysteine and methionine suggested a possible role for tissue amino acid levels in the regulation of liver enzyme concentration. If low protein diets altered amino acid levels only in the liver, this might explain the absence of changes in 3-P-glycerate dehydrogenase in other tissues. The effects of low protein diets on specific tissue amino acid levels have not been recorded previously. Therefore, studies correlating enzyme levels with tissue amino acid content were undertaken.

Alterations in amino acid levels in rats fed 2% casein were limited to the liver, the only tissue in which changes in 3-P-glycerate dehydrogenase occurred. A decline in hepatic cystine, methionine and taurine content in the rats fed 2% casein was partially prevented by addition of either cysteine or methionine to the diet. Cystine supplements increased cystine and taurine levels and methionine supplements raised the concentration of methionine and taurine. Thus, the 3-P-glycerate dehydrogenase level is not correlated with either cystine or methionine concentration, alone. The data suggest that both cysteine and methionine may exert independent effects, or that some common derivative of both is most critical in this correlation.

These studies are compatible with an association of hepatic 3-P-glycerate dehydrogenase levels with the tissue content of sulfur-containing amino acids or one of their derivatives. Previous studies suggest that the dietary-induced change in 3-P-glycerate dehydrogenase is caused by increased enzyme synthesis (3, 5). Thus, changes in tissue amino acid concentration may participate in the regulation of enzyme synthesis. A similar process has been regularly observed in microorganisms but is not reported for mammalian systems. No definitive conclusion on mechanism can be made from these correlative data, however, and these effects may be related independently to other factors, such as hormone levels, or the concentration of carbohydrate intermediates.

A decrease in liver serine and glycine content occurred when cysteine or methionine supplements were added to the low protein diet. This observation is evidence for the physiological significance of changes in hepatic 3-P-glycerate dehydrogenase levels. The supplements of sulfur-containing amino acids prevent the increase of this enzyme in response to low

protein diets. A reduced endogenous synthesis of serine and glycine is an apparent consequence, manifested by the lower concentration of serine and glycine in the liver. The increase in 3-P-glycerate dehydrogenase levels noted in rats fed 2% casein diets apparently permits increased serine biosynthesis and prevents a marked decline in hepatic serine and glycine concentration. The close correlation of changes in alanine concentration with those of serine and glycine suggests that alanine concentration may depend on the availability of serine in the protein-depleted rat liver. Under these conditions, alanine might be a significant source of serine by transamination of hydroxypyruvate (12).

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Effect of Ascorbic Acid on Fluoride Uptake in the Polymorphonuclear Leukocyte of the Guinea Pig [1]

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ABSTRACT Radiotracer fluorine-18 was produced, isolated, and used as a physiological tracer in an investigation of the effect of ascorbic acid on the uptake of fluoride by exudate leukocytes of the guinea pig. It was possible to measure fluoride uptake to within ± 0.0005 $\mu g/10^7$ cells by this method. When the leukocytes were incubated at concentrations of 2.4 ppm and 1.2 ppm fluoride, the cells containing high levels of ascorbic acid (more than 0.9 μg ascorbic acid/10^7 cells) took up significantly more fluoride than those containing low levels (less than 0.2 μg ascorbic acid/10^7 cells). Addition of ascorbic acid to the medium did not cause an increase in the uptake of fluoride. With incubation in 2.4 ppm fluoride both types of cells took up approximately twice as much fluoride as they did at 1.2 ppm.

Little is known about the effect of ascorbic acid on fluoride metabolism. In a study by Elliott and Smith (1) of ascorbic acid metabolism in exudate leukocytes from guinea pigs, fluoride was found to decrease ascorbic acid loss from cells during incubation and to stimulate its uptake. The vitamin's effect on metabolism of fluoride has been investigated in whole animals. In experiments with guinea pigs Muhler (2) concluded that ascorbic acid increased retention of fluoride. Stookey et al. (3) found that when ascorbic acid was administered in addition to fluoride the rate of absorption of fluoride in guinea pig intestine was not influenced during the first 4 hours. After 12 hours, increased absorption of fluoride was noted.

Studies by Venkateswarlu and Narayanarao (4) indicated that ascorbic acid had no influence on fluoride metabolism. In a later publication (5), these workers reported that ingestion of fluoride had no influence on excretion and storage of ascorbic acid in the guinea pig and suggested that it, in turn, had no influence on retention and excretion of fluoride.

This report presents results of experiments designed to investigate the effect of ascorbic acid on uptake of fluoride using the leukocyte system described by Elliott and Smith (1). It was found that levels at which fluoride is taken up by the leukocytes could not be detected using the chemical methods of Elliott and Smith (6). Radiochemical procedures, which are more sensitive, were therefore developed and used.

METHODS

Production and isolation of fluorine-18. Lithium carbonate packed in quartz vials approximately 3 mm in diameter was bombarded with thermal neutron flux of 1.5 to 1.8×10^{13} neutrons/cm^2/second for at least 5 hours. A yield of approximately 1 to 2 mCi of fluorine-18 from 1 g of irradiated lithium carbonate was obtained by the following nuclear reactions:

$$^{6}_{3}\mathrm{Li} + {}^{1}_{0}\mathrm{n} \longrightarrow {}^{3}_{1}\mathrm{H} + {}^{4}_{2}\mathrm{He}$$

$$^{3}_{1}\mathrm{H} + {}^{16}_{8}\mathrm{O} \longrightarrow {}^{18}_{9}\mathrm{F} + {}^{1}_{0}\mathrm{n}$$

After irradiation fluorine-18 was isolated by a modification of the method described by Banks (7). Ten micrograms of sodium fluoride were added as carrier and the irradiated lithium carbonate was dis-

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solved in concentrated nitric acid. Addition of 5% lead acetate and concentrated hydrochloric acid precipitated lead as chlorofluoride and chloride. The precipitate was dissolved in a 25% sodium hydroxide solution and the resulting solution was acidified with 10% sulfuric acid. The lead was removed by precipitation as lead sulfate and subsequent centrifugation. The supernatant which contained the fluorine-18 was passed through a Dowex 50W-X8 anion column to reduce sodium-24 contamination to less than 5%. For use as a tracer in a physiological system the pH of the column eluant was adjusted to 7.4 and an appropriate amount of sodium chloride was added to make the solution isotonic.

Animals and diets. Adult male guinea pigs 1 to 2 years of age (800–1200 g) were used in these studies. All animals were offered an ad libitum diet of rabbit ration.[5] An average daily consumption of 35 g by the animals was determined by placing several of them in individual cages and measuring feed intake. Analyses of samples of rabbit ration gave ascorbic acid values ranging from 6.7 mg/100 g for fresh ration to 2.3 mg/100 g for aged ration with an average value, including all analyses, of 4.7 mg/100 g. On this basis animals consuming rabbit ration only would ingest approximately 2 mg ascorbic acid/day and would eventually die of scurvy. These animals were assumed to be deficient in ascorbic acid intake and the ascorbic acid content of their exudate leukocytes would lie below 0.2 μg/10^7 cells (0.156 ± 0.017 SE μg ascorbic acid/10^7 cells in 9 experiments) (1). The animals showed no visible external signs of scurvy during the course of the experiments although their cells contained little ascorbic acid. To produce leukocytes containing high levels of ascorbic acid, animals consumed rabbit ration supplemented with 10 mg ascorbic acid/animal/day administered orally by pipet. Increasing this to 100 mg/day for 5 days prior to experimental use of the animals gave leukocytes containing more than 0.9 μg ascorbic acid/10^7 cells (1.05 ± 0.042 SE μg ascorbic acid/10^7 cells in 8 experiments) (1). A more rigid control of ascorbic acid intake was not felt to be necessary for these experiments.

General experimental procedures. Suspensions of guinea pig leukocytes were prepared as outlined in detail by Elliott and Smith (1). Briefly, three animals were injected intraperitoneally with 100 ml of 0.1% sodium caseinate in normal saline. This solution, left in the animals for sixteen hours, was then drained from the peritoneal cavity and all exudates pooled. The exudate fluid, rich in leukocytes, was centrifuged and the cells resuspended in a balanced salt solution (8). The suspension was allowed to stand for a few minutes so that clumps of cells would settle out; it was then decanted, centrifuged, and resuspended. A suspension free of clumps of cells and containing approximately 2×10^7 cells/ml was finally obtained. Cells in suspensions were counted using a hemocytometer.

A smear of the suspension was prepared with Wright's stain to check the types of cells present and contamination with erythrocytes. The proportion of polymorphonuclear leukocytes was always 90% or better of the total cells present. Suspensions containing more than 3% erythrocytes were not used. Four milliliter aliquots of the homogeneous cell suspension were pipetted into 30-ml polyethylene bottles suspended in a constant temperature bath. Four samples were used in each experiment. To samples 1 and 2, 2.4 ppm fluoride and tracer were added; to samples 3 and 4, 1.2 ppm fluoride and tracer were added. In addition samples 2 and 4 contained 3×10^{-4} moles/liter of ascorbic acid. All samples were shaken throughout the preparative and incubation steps.

Following a two-hour incubation the contents of the bottles were transferred to 15-ml graduated centrifuge tubes; each polyethylene bottle was washed with 2 ml of balanced salt solution (8), washings were added to the tubes, and the resulting suspensions were centrifuged. An aliquot of the supernatant was pipetted into a counting tube. The rest was discarded. The inside of the tube containing the cells was wiped with tissue to remove any adhering supernatant, and 2 ml of balanced salt solution was added. The cells, supernatant, and reference tracer solutions were

[5] Purina Rabbit Chow, Ralston Purina Company, St. Louis.

counted in a well-type gamma-scintillation counter, decay curves were plotted, and a correction for sodium-24 contamination was made. The ratios of counts of the packed cells to the total counts added were used to calculate the fluoride taken up by the cells.

RESULTS AND DISCUSSION

A satisfactory fluoride tracer was obtained using this method of fluorine-18 production, isolation, and subsequent correction for sodium-24. Using fluorine-18 it was possible to detect uptake of fluoride by the leukocytes in quantities as little as $\pm$ 0.0005 μg of fluoride per 10^7 cells.

Three main observations can be stated from the experimental results presented in table 1. When ascorbic acid was varied only in vitro no difference in the uptake of fluoride between the cells exposed to ascorbic acid and those not exposed could be noted. The results were analyzed statistically and no significant difference was indicated at the 95% level of confidence. When the uptake of fluoride by cells deficient in ascorbic acid was compared with those with high levels of ascorbic acid it was noted that the latter took up markedly more fluoride. The difference was statistically significant at the 95% confidence level. When the exudate leukocytes were exposed to 2.4 ppm of fluoride they took up approximately twice as much fluoride as those exposed to 1.2 ppm of fluoride.

Wallace-Durbin (9) concluded that the cell membrane of rat tissue is freely permeable to fluoride. Stookey et al. (10) suggested that an active transport system for fluoride does not exist in rat intestine and that absorption takes place by simple diffusion. This suggestion was based on experiments which demonstrated the following: enzyme inhibitors do not affect the rate of absorption of fluoride; alteration in temperature had little effect on the rate; fluoride would not move against a concentration gradient; and absorption was directly proportional to the area of gut exposed.

In our experiments increase in the concentration of fluoride from 1.2 to 2.4 ppm resulted in uptake of approximately twice as much fluoride. This adds support to the suggestion that uptake of fluoride is a diffusion process and not an active transport system.

Addition of ascorbic acid during the incubation had no effect on the uptake of fluoride by cells with high and low levels of endogenous ascorbic acid. A similar relation was observed by Stookey et al. (3) who found ascorbic acid affected fluoride uptake only after twelve hours. It appears that the effect of ascorbic acid is delayed and therefore may be an indirect one. This argument is supported by the observation that endogenous levels of the vitamin have an effect on fluoride uptake after two

TABLE 1

In vitro uptake of fluoride by exudate leukocytes of the guinea pig [1]

		Leukocytes deficient in ascorbic acid	Leukocytes containing high levels of ascorbic acid
		μg fluoride/10^7 cells/2 hours	
I	2.4 ppm fluoride in incubation medium		
	a) Ascorbic acid added to medium	0.012 $\pm$ 0.0001 [2] (4) [3]	0.024 $\pm$ 0.0035(2)
	b) No ascorbic acid added to medium	0.011 $\pm$ 0.0000 (3)	0.026 $\pm$ 0.0035(5)
	a) and b) combined	0.011 $\pm$ 0.0006	0.026 $\pm$ 0.0026
II	1.2 ppm fluoride in incubation medium		
	c) Ascorbic acid added to medium	0.006 $\pm$ 0.0005 (3)	0.011(1)
	d) No ascorbic acid added to medium	0.007 $\pm$ 0.0005 (3)	0.016 $\pm$ 0.0010(3)
	c) and d) combined	0.006 $\pm$ 0.0003	—

[1] In those experiments with ascorbic acid in the medium, ascorbic acid concentration was 3.0×10^{-4} M.
[2] The standard error (SE) of the mean is quoted for each result.
[3] Number of experiments shown in parentheses.

hours incubation. It might be speculated that the effect of ascorbic acid was related to permeability of cell membrane. Perhaps cells containing high levels of ascorbic acid had membranes in a condition which permitted much freer diffusion of fluoride than was the case of cells with low levels of endogenous ascorbic acid.

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Effects of a Potassium Deficiency on Calcification of Bone in Chicks [1,2]

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ABSTRACT The effects of a potassium deficiency on bone formation were studied in chicks aged 1 or 10 days which were fed various levels of potassium for an experimental period of 7 days. The older chicks had been maintained on a marginally adequate level of potassium (2800 ppm) during the 10-day preexperimental period. Paired-feeding studies were also conducted to determine the effects of the reduced feed consumption observed with a potassium deficiency on the various parameters investigated. A potassium deficiency resulted in a reduction in bone ash in chicks of both age groups. In the younger chicks, the phosphorus content of the bone ash was reduced by the potassium deficiency; however, a comparable reduction in bone ash phosphorus was not apparent with the older chicks. The calcium content of the bone ash was not affected by the potassium deficiency in either age group. Paired-feeding was without effect in altering bone ash or the calcium and phosphorus content of bone ash. A reduction in the potassium content and an increase in the sodium content of the bone ash was observed in the potassium-deficient chicks. The magnesium content of the bone ash was not affected by the potassium deficiency. The incorporation of ^{32}P into femurs 4 hours postintraperitoneal injection was reduced in potassium-deficient chicks of both ages as compared with chicks receiving the diet adequate in potassium either ad libitum or pair-fed. In general, ^{45}Ca incorporation was also reduced, although the results were not as consistent nor of as great a magnitude as with ^{32}P. Paired-feeding did not affect ^{32}P incorporation into bone, and the effect on ^{45}Ca incorporation was inconsistent. In contrast, the concentrations of ^{45}Ca and ^{32}P in skeletal muscle 4 hours postinjection were increased in potassium-deficient chicks, whereas no differences were observed in the concentrations of these isotopes in heart muscle or liver.

The influence of potassium on bone mineralization has received relatively little attention. Gillis (1) reported a reduction in bone ash in potassium-deficient chicks. Later, Gillis (2) suggested that the reduction in calcification due to a potassium deficiency was mediated through an influence on phosphorus rather than calcium metabolism. Cuisinier-Gleizes et al. (3) found that bones from potassium-deficient rats had a higher moisture content, but that the mineral content of the bone was significantly lowered only in rats which survived for more than eighty days.

The present study was conducted to investigate further the influence of potassium on calcium and phosphorus metabolism with special emphasis on bone formation.

EXPERIMENTAL PROCEDURE

Either 1-day-old or 10-day-old Hubbard White Mountain male chicks were fed the experimental diets for a period of 7 days. The older chicks had been maintained on a marginally adequate level of potassium (2800 ppm) during the 10-day preexperimental period. The chicks placed on the experimental diets at 1 and 10 days of age will hereafter be referred to as 7 and 17 days old, respectively.

The basal diet used in these studies contained 30% isolated soybean protein, 54.25% glucose monohydrate, 5% soybean oil, 3% cellulose, 0.5% DL-methionine, 0.3% glycine, 0.015% butylated hydroxytoluene, 5.73% mineral mix (4) and 1.2% vitamin premix (4). The various levels of potassium were obtained by the substitution of an appropriate amount of

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[2] Presented in part at the 50th Annual Meeting of the American Institute of Nutrition, Atlantic City, New Jersey, 1966. Federation Proc., 25: 610 (abstract).

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potassium carbonate for an equal amount of glucose monohydrate.

Deionized water was supplied ad libitum throughout the experimental period. With the exception of the paired-feeding experiments, feed was also supplied ad libitum throughout the experiment. The pair-fed chicks received an amount of feed, adequate in potassium (4000 ppm), equal to that consumed by chicks fed a deficient diet on the preceding day.

In the bone ash studies, the bones were excised at the end of the 7-day experimental period, cleaned of adhering tissue and extracted for 12 hours with 95% ethanol in a Soxhlet apparatus followed by a 12-hour extraction with petroleum ether. The moisture-free, fat-free bones were then ashed individually in a muffle furnace at 600° for 12 hours, and the ash expressed as a percent of the moisture-free, fat-free bone. The bone ash was then dissolved and made to volume in 1 N HCl for elemental analyses. Individual analyses were conducted for phosphorus by the method of Fiske and Subbarow (5), and calcium, potassium, sodium and magnesium were determined by atomic absorption spectrophotometry.[4]

For the isotope experiments, 0.5 ml of an isotonic saline solution containing both calcium-45 (specific activity 8.1 mCi/mg) and phosphorus-32 (specific activity 30.3 mCi/mg) was injected intraperitoneally. The activity of the injected dose was 0.5 μCi ^{45}Ca and 0.5 μCi ^{32}P for experiments 5, 6 and 7, 4.0 μCi ^{45}Ca and 4.0 μCi ^{32}P for experiment 8, and 2.0 μCi ^{45}Ca and 2.0 μCi ^{32}P for experiments 9, 10 and 11. The femurs and soft tissues were excised four hours postinjection and weighed immediately on a torsion balance. The samples were ashed for 12 hours in a muffle furnace at 600° and the ash dissolved in 3 ml of 1 N HCl. A 1 ml aliquot of the dissolved ash was transferred to a counting vial, 20 ml of scintillation fluid (4) added and the samples counted in a dual channel liquid scintillation counter.[5] Since the injected dose remained constant regardless of body weight rather than adjusted to the weight of each individual chick, a mathematical correction for body weight was made and the results expressed as counts per minute per milligram of fresh tissue times chick weight (g).

All data were subjected to analysis of variance as outlined in Steel and Torrie (6) and mean differences were compared by the use of the Newman-Keuls sequential test. The 0.01 level of probability was used for all subsequent statements of statistically significant differences.

RESULTS AND DISCUSSION

As reported previously (7), the first symptoms observed upon feeding either 1-day-old or 10-day-old chicks a diet grossly deficient in potassium included a rapid reduction in food consumption and growth rate. The most severe symptoms were observed in the younger chicks. This rapid reduction in food consumption necessitated the use of paired-feeding to determine whether the differences observed resulted from a potassium deficiency per se, or merely a reduction in the intake of all nutrients.

Initial experiments were concerned with the effects of a potassium deficiency on bone ash and the concentrations of certain elements in bone ash (table 1). A significant reduction in bone ash was observed in 7-day-old chicks fed the potassium-deficient diet as compared with chicks fed the diet adequate in potassium, either ad libitum or pair-fed. There was no significant effect of paired-feeding on bone ash values. In general, the results obtained with 17-day-old chicks were similar, although the magnitude of the differences between chicks fed diets deficient and adequate in potassium were not as great, and in one experiment, the difference between the deficient and pair-fed chicks was not statistically significant.

There was no significant effect of a potassium deficiency on the calcium content of the bone ash regardless of the age of the chicks. There was a significant reduction, however, in the phosphorus content of the bone ash from 7-day-old chicks fed the potassium-deficient diet as compared with chicks fed the control diet either ad libitum or pair-fed. Paired-feeding had no effect on the phosphorus content of the

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

[5] Model 314EX (Tri-Carb) Packard Instrument Company, Inc., Downers Grove, Illinois.

TABLE 1

Effect of a potassium deficiency on bone ash and mineral composition of bone ash [1]

	Bone ash [2]	Ca	P	K	Na	Mg
			% of bone ash			
Exp. 1 — 7 days [3]						
550 ppm K	32.31 [a]	35.93 [a]	14.69 [a]	—	—	—
4000 ppm K	38.40 [b]	36.23 [a]	16.63 [b]	—	—	—
4000 ppm K (PF) [4]	37.40 [b]	36.11 [a]	16.02 [b]	—	—	—
Exp. 2 — 7 days [3]						
550 ppm K	32.10 [a]	36.98 [a]	14.07 [a]	0.73 [a]	2.57 [a]	6.82 [a]
4000 ppm K	37.60 [b]	37.86 [a]	17.07 [b]	0.88 [b]	1.93 [b]	6.48 [a]
Exp. 3 — 17 days [5]						
550 ppm K	39.12 [a]	35.57 [a]	17.19 [a]	—	—	—
4000 ppm K	41.25 [b]	33.31 [a]	17.01 [a]	—	—	—
4000 ppm K (PF) [4]	39.87 [ab]	33.99 [a]	17.08 [a]	—	—	—
Exp. 4 — 17 days [5]						
550 ppm K	33.30 [a]	36.65 [a]	18.44 [a]	0.79 [a]	1.94 [a]	6.92 [a]
4000 ppm K	36.85 [b]	34.48 [a]	19.11 [a]	0.89 [a]	1.80 [a]	6.77 [a]

[1] Values are means from 10 chicks/group. Means with different superscripts in a vertical column within experiments are significantly different ($P < 0.01$).
[2] Expressed as percent of dry, fat-free bone.
[3] Fed the experimental diets from 1-day-old to 7 days of age.
[4] Pair-fed the amounts of feed consumed by the low K group on the preceding day.
[5] Fed the experimental diets from 10 days of age to 17 days of age.

bone ash. This influence of a potassium deficiency on the phosphorus content of the bone ash was not apparent in 17-day-old chicks.

The potassium deficiency significantly reduced the potassium content and significantly increased the sodium content of bone ash from 7-day-old chicks. Although the same trend was apparent in 17-day-old chicks, the differences were not significant. The potassium deficiency was without effect in altering the magnesium content of the bone ash.

Previous studies of ^{45}Ca and ^{32}P incorporation into bone at intervals from 2 to 48 hours after intraperitoneal administration of the isotopes have indicated that maximal incorporation had occurred by 4 hours and that only slight changes in radioactivity occurred between this time and 48 hours postinjection.[6] The effects of dietary potassium on the incorporation of ^{45}Ca and ^{32}P into femurs 4 hours post-intraperitoneal injection are presented in tables 2 and 3. In all experiments, chicks fed a diet deficient in potassium incorporated significantly less ^{32}P into their femurs than chicks receiving adequate levels of potassium either ad libitum or pair-fed. No differences were observed in ^{32}P incorporation between chicks fed the diet adequate in potassium ad libitum and those receiving the same diet pair-fed with the potassium-deficient group. In general, ^{45}Ca incorporation into femurs was also reduced, although the results were not as consistent nor of as great a magnitude as for ^{32}P. Since ^{45}Ca was not affected as greatly as ^{32}P, the ^{45}Ca-to-^{32}P ratio in the femurs was increased in the potassium-deficient chicks. The results with paired-feeding were inconsistent; in two experiments there appeared to be a reduction in ^{45}Ca incorporation with pair-fed chicks, but in another experiment there was an increase in incorporation of ^{45}Ca.

These results, along with the bone ash data, agree with the conclusions of Gillis (2) that the primary effect of potassium on calcification involves phosphorus metabolism rather than calcium metabolism.

A potassium deficiency resulted in a significant increase in the concentrations of ^{45}Ca and ^{32}P found in skeletal muscle 4 hours postintraperitoneal injection (table 4). Paired feeding had no effect on the concentration of these isotopes in skeletal muscle. The effect of a potassium deficiency noted in skeletal muscle was not observed in heart muscle nor liver (table

[6] J. C. Rogler and H. E. Parker, 1965, unpublished data.

TABLE 2

Effect of potassium on ^{45}Ca and ^{32}P incorporation into femurs [1]

Exp. no. and chick age	Dietary K, ppm			
	800	1600	2800	4000
Exp. 5 — 7 days [2]				
^{45}Ca	4713 [a]	5217 [ab]	5714 [b]	—
^{32}P	2450 [a]	2646 [a]	3705 [b]	—
Exp. 6 — 7 days [2]				
^{45}Ca	6402 [a]	—	7181 [b]	7329 [b]
^{32}P	2178 [a]	—	2594 [b]	3068 [b]
Exp. 7 — 17 days [3]				
^{45}Ca	2230 [a]	—	2224 [a]	2167 [a]
^{32}P	737 [a]	—	872 [b]	909 [b]
Exp. 8 — 17 days [3]				
^{45}Ca	18,233 [a]	—	—	24,654 [b]
^{32}P	10,070 [a]	—	—	17,219 [b]

[1] Expressed as cpm/mg of fresh bone × chick wt (g). Average of 8 chicks/group. Values listed horizontally with different superscripts are significantly different ($P < 0.01$).
[2] Fed the experimental diets from 1-day-old to 7 days of age.
[3] Fed the experimental diets from 10 days of age to 17 days of age.

TABLE 3

Influence of dietary potassium and paired feeding on ^{45}Ca and ^{32}P incorporation into femurs [1]

Exp. no. and chick age	Dietary K, ppm		
	800	4000	4000 PF [2]
Exp. 9 — 7 days [3]			
^{45}Ca	7201 [a]	7500 [a]	6755 [b]
^{32}P	3145 [a]	5200 [b]	5153 [b]
Exp. 10 — 17 days [4]			
^{45}Ca	15,420 [a]	17,370 [b]	16,550 [c]
^{32}P	9635 [a]	13,855 [b]	13,850 [b]
Exp. 11 — 17 days [4]			
^{45}Ca	11,786 [a]	12,755 [ab]	13,238 [b]
^{32}P	6378 [a]	13,254 [b]	12,579 [b]

[1] Expressed as cpm/mg of fresh bone × chick wt (g). Average of 8 chicks/group. Values listed horizontally with different superscripts are significantly different ($P < 0.01$).
[2] Fed the experimental diets from 1-day-old to 7 days of age.
[3] Pair-fed the amount of feed consumed by the low K group on the preceding day.
[4] Fed the experimental diets from 10 days of age to 17 days of age.

4) where the concentrations of ^{45}Ca and ^{32}P were remarkably constant regardless of potassium level or paired feeding.

It may be reasoned that the increase in ^{45}Ca and ^{32}P in skeletal muscle is a reflection of the reduced incorporation of these isotopes into bone, since bone is the site of most of the body calcium and phosphorus. Even a slight reduction in incorporation into bone would result in the availability of greater quantities of an injected dose for concentration in other tissues. The observations that a potassium deficiency does not significantly increase total calcium and phosphorus in skeletal muscle lend credence to this theory (0.98 and 1.04% Ca and 4.17 and 3.94% P in the ash for deficient and control chicks, respectively). One would expect that response of heart muscle and liver would be similar to that of skeletal muscle. It has been observed that the potassium content of skeletal muscle is reduced significantly by feeding a potassium-deficient diet for 7 days, whereas no effect of a deficiency could be shown in the potassium content of heart muscle and liver (7). Thus, it is possible that the observed differences in ^{45}Ca

TABLE 4

Effect of dietary potassium on ^{45}Ca and ^{32}P incorporation into skeletal muscle, heart muscle and liver [1]

Tissue and exp. no.	Dietary K, ppm		
	800	4000	4000 PF [2]
Skeletal muscle			
Exp. 9 [3]			
^{45}Ca	431 [a]	195 [b]	206 [b]
^{32}P	1166 [a]	851 [b]	780 [b]
Exp. 10			
^{45}Ca	240 [a]	160 [b]	176 [b]
^{32}P	2002 [a]	1122 [b]	1413 [b]
Exp. 11			
^{45}Ca	200 [a]	114 [b]	116 [b]
^{32}P	2107 [a]	1270 [b]	1571 [b]
Heart muscle			
Exp. 9			
^{45}Ca	136 [a]	138 [a]	144 [a]
^{32}P	2734 [a]	2784 [a]	2892 [a]
Liver			
Exp. 9			
^{45}Ca	452 [a]	456 [a]	426 [a]
^{32}P	2986 [a]	3126 [a]	3290 [a]

[1] Expressed as cpm/mg of fresh tissue × chick wt (g). Average of 8 chicks/group. Values listed horizontally with different superscripts are significantly different ($P < 0.01$).
[2] Pair-fed the amount of feed consumed by the low K group on the preceding day.
[3] Tissues from the same chicks in a given experiment for which bone data are presented in table 3.

and ^{32}P concentrations may be associated with the potassium content of the tissue rather than a reflection of reduced incorporation into bone.

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Rat Intestinal Dipeptidase Activity During Oral Neomycin and Gluten Administration

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ABSTRACT The mechanism of neomycin-induced azotorrhea and the role of dietary gluten were studied by measuring intestinal dipeptidase activity in rats given neomycin with or without dietary gluten. Rats fed a 10%-wheat-gluten–8%-casein diet or 18% casein (gluten-free) diet were given 100 mg neomycin daily by gastric tube for 7 days. Control groups were fed the appropriate diet, but no neomycin. Homogenates of intestinal mucosa were assayed for dipeptidase activity, using as substrates: glycyl-alanine, glycyl-L-leucine, glycyl-L-valine, DL-alanyl-glycine, glycyl-L-isoleucine, L-leucyl L-leucine, and DL-alanyl-L-leucine. Mean dipeptidase activity was significantly depressed only in the rats fed 10% gluten plus neomycin. Rats given neomycin and fed an 18%-casein (gluten-free) diet had normal intestinal dipeptidase activity. Neomycin inhibited gain in body weight in both experimental groups. The observed depression of dipeptidase activity may explain in part the increased fecal nitrogen excretion observed in man during neomycin administration. The experimental data indicate that neomycin in the presence of gluten partially inhibits intestinal dipeptidase activity.

Neomycin produces a generalized malabsorption syndrome in man resulting in increased fecal excretion of fat, saccharides, electrolytes, and nitrogen (1–3). Neomycin may impair fat absorption by interfering with chylomicron formation (4) and disturbing intraluminal events in lipid digestion (5, 6).[1] Reversible depression of intestinal lactase activity and monosaccharide absorption explains the sugar malabsorption produced by neomycin (7–9). Except for the observation of Jacobson et al. (1) that there is an increase in fecal nitrogen following the oral administration of neomycin, no studies on the effect of neomycin on protein digestion and absorption have been reported. Therefore, the present study was undertaken to determine the effects of neomycin on the final step in protein digestion, namely the hydrolysis of small peptides into their component amino acids by intestinal mucosal cell peptidases. Because a gluten-free diet may ameliorate neomycin-induced malabsorption (10),[2] the role of dietary gluten was investigated.

EXPERIMENTAL

Materials. A 0.15 M phosphate buffer according to the method of Sørensen, was used to prepare aqueous dipeptide solutions (table 1).[3] The pH used was found to be optimum over a range of 6 to 8. No metal ions were added. The purity of all dipeptides and amino acids was confirmed by thin-layer chromatography on silica gel-G with propan-2-ol–formic acid–water (40:2:10, v/v), and 0.1% ninhydrin for development of the spots.

TABLE 1

Molar concentration and pH of dipeptide solutions

Dipeptide solution	Molar conc	pH
Glycyl-alanine	0.06	7.6
Glycyl-L-leucine	0.04	7.5
Glycyl-L-valine	0.03	7.5
DL-Alanyl-glycine	0.06	7.6
Glycyl-L-isoleucine	0.04	7.8
L-Leucyl-L-leucine	0.03	7.8
DL-Alanyl-L-leucine	0.03	7.6

Assay of dipeptidase activity. The dipeptidase assay described by Josefsson and Lindberg (11) was modified as follows: 0.04 ml of enzyme solution was incubated at 40° with 0.1 ml dipeptide solu-

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[3] Dipeptides and amino acids were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, except for DL-alanine, which was obtained from Sigma Chemical Company, St. Louis.

tion and 0.2 ml buffer. After 2.5 minutes, hydrolysis was interrupted by the addition of 2.6 ml ethanol–water (99:1, v/v). The solutions were allowed to stand for 10 minutes and then centrifuged for 30 minutes at 3000 rpm. The supernatant was transferred to quartz cuvettes of 1 cm light path and the absorbency measured at 22 mμ in a spectrophotometer.[4] Identically treated samples containing 0.1 ml of the corresponding amino acid solution in equimolar concentration instead of the dipeptide solution served as blanks. Total protein was determined by the method of Lowry et al. (12).

The results are expressed in units of dipeptidase activity. One unit is the activity hydrolyzing 1 μmole of dipeptide per minute at 40°. Nitrogen was estimated from the protein determination assuming that 16% of the total protein is nitrogen.

Plan of experiments. Two separate experiments were carried out. Experiment 1 was designed to observe the effects of neomycin plus gluten on intestinal dipeptidase activity. Female rats of the Holzman strain were housed in individual metabolic cages. Twenty animals weighing approximately 360 g were maintained with a purified gluten diet containing 10% wheat gluten,[5] 8% casein,[6] 68% sucrose, 10% corn oil and salt mixture, USP XIV 4%, fortified with vitamins [7] for 2 weeks. At the end of the first week one-half the animals were given 50 mg neomycin in 1 cm^3 of water by gastric tube, twice a day. On day 14, after a 24-hour fast (except for drinking water and neomycin) the animals were killed by stunning exactly 2 hours after the final dose of neomycin. The small intestine was promptly removed and opened. The mucosa was rinsed with ice-cold normal saline, blotted and a 10 cm segment scraped off with a chilled microscope slide beginning at the ligament of Treitz and extending 10 cm distally. The mucosa was homogenized for 5 minutes in 5 ml of 0.1 M sodium chloride solution at 4° by hand with a glass tissue grinder. The homogenate was allowed to stand for 30 minutes, and then centrifuged at 27,000 × *g* for 30 minutes at 4°. The clear supernatant was used for enzyme activity and total protein determination.

The experiment was carried out over a period of several weeks: the starting dates were staggered so that one neomycin-treated and one control animal were killed at the same time.

Experiment 2 was designed to observe the effects of neomycin without gluten on intestinal dipeptidase activity. Twenty female rats weighing approximately 260 g were used. Gluten was replaced by casein so that the final casein concentration was 18% instead of 8%. Otherwise the experimental design was identical to that described for experiment 1.

RESULTS

Mean dipeptidase activity for the groups fed a 10%-gluten–8%-casein diet is shown in figure 1, and for the groups fed an 18%-casein–gluten-free diet in figure 2. Mean dipeptidase activity was significantly decreased ($P < 0.05$) only in the rats fed gluten and neomycin. In contrast, none of the rats given neomycin, but fed a gluten-free (18% casein) diet, showed a significant depression of intestinal dipeptidase activity. The mean dipeptidase activity for each dipeptide did not differ significantly in the control groups fed 18% casein or 10%-gluten–8%-casein diets ($P > 0.10$).

All animals given neomycin developed persistent watery diarrhea after the fifth day. Weight gain (mean ± SD) during 14 days was 6.3 ± 5.1 g for the rats given gluten plus neomycin and 13.3 ± 4.4 g for the gluten-fed controls ($P < 0.01$). Mean weight gain for the rats given 18% casein plus neomycin but no gluten was 11.7 ± 3.4 g and 18.4 ± 2.9 g for those fed the same diet without neomycin ($P < 0.001$). The data clearly indicate that neomycin

[4] Zeiss Spectrophotometer, Carl Zeiss Works, Stuttgart, Germany.

[5] Nitrogen content, 13.6%.

[6] Nitrogen content, 14.5%.

[7] The vitamin mixture contained: (in g/45.5 kg) vitamin A (200,000 units/g), 4.5; vitamin D (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; and Ca pantothenate, 3.0; (and in mg/45.5 kg diet) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35. The mineral mixture contained: (in percent) cupric sulfate, 0.078; ferric ammonium citrate, 15.280; manganese sulfate, 0.200; ammonium alum, 0.093; potassium iodide, 0.042; sodium fluoride, 0.507; calcium carbonate, 6.860; calcium citrate, 30.830; calcium biphosphate, 11.280; magnesium carbonate, 3.520; magnesium sulfate, 3.830; potassium chloride, 12.470; dibasic potassium phosphate, 21.880; and sodium chloride, 7.710. Vitamins, minerals and diets were obtained from Nutritional Biochemicals Corporation.

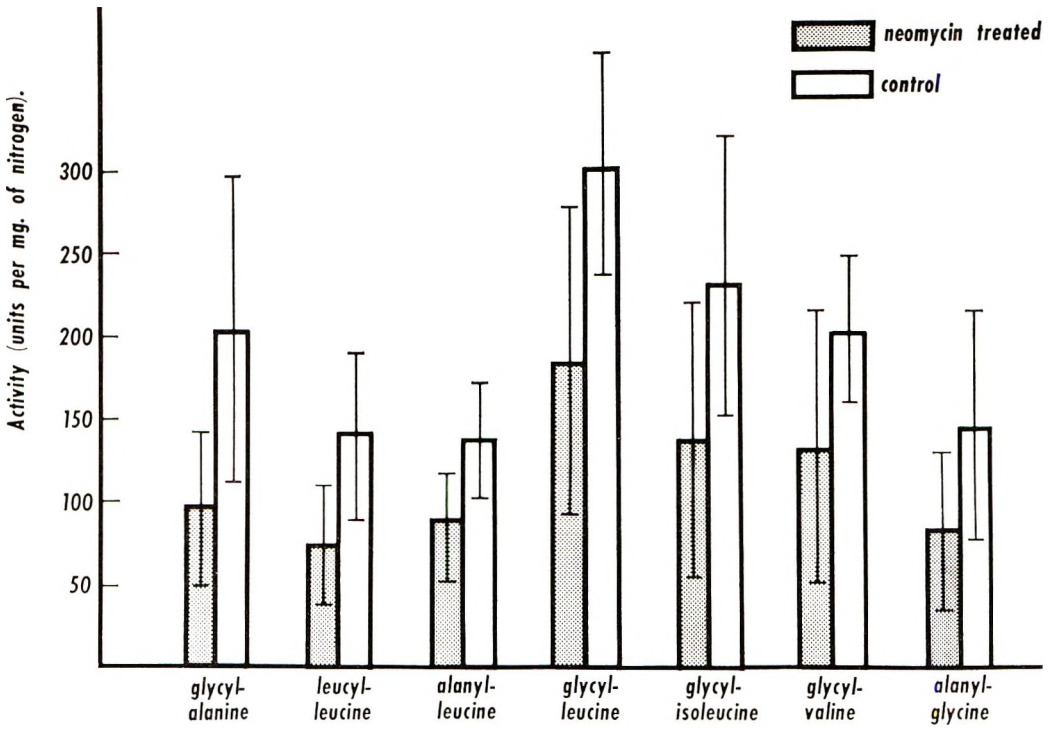

Fig. 1 Effects of neomycin on intestinal dipeptidase activity of rats fed gluten. Each bar shows the mean value and standard deviation obtained from 10 animals. The differences are significant: ($P < 0.01$: glycyl-L-alanine, glycyl-L-leucine, L-leucyl-L-leucine; $P < 0.02$: DL-alanyl-L-leucine, glycyl-L-isoleucine; $P < 0.05$: glycyl-L-valine, DL-alanyl-glycine).

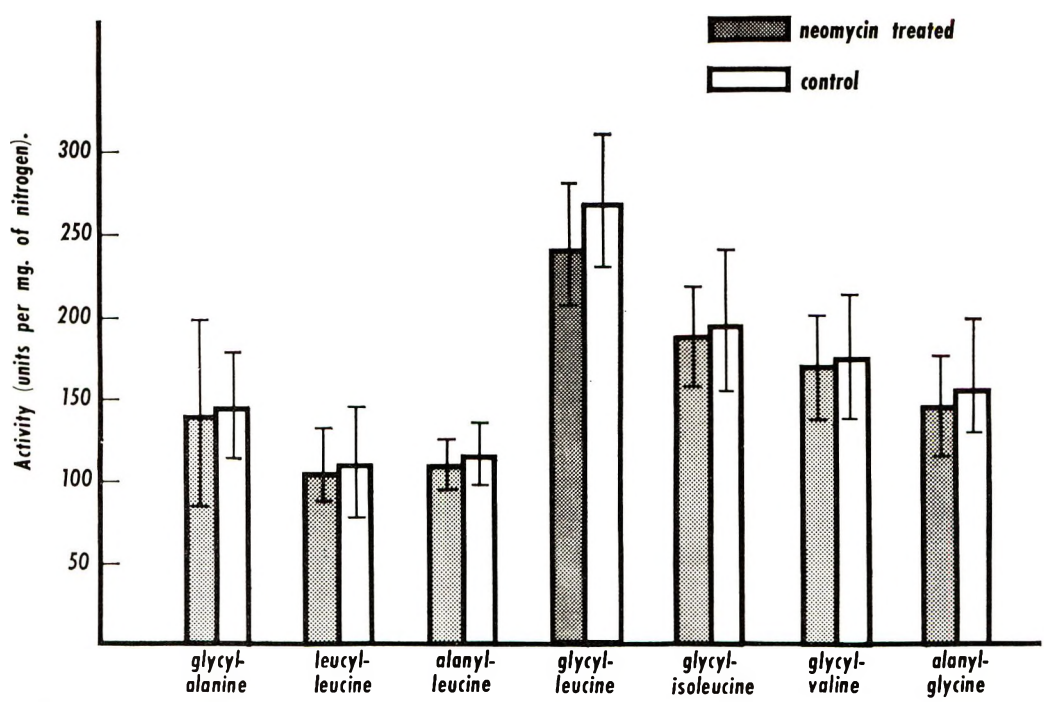

Fig. 2 Effects of neomycin on intestinal dipeptidase activity of rats fed a gluten-free diet. Each bar shows the mean value and standard deviation obtained from 10 animals. The differences are not significant ($P > 0.10$).

had a significant influence on body weight gain, a consequence, at least in part, to the persistent watery diarrhea which occurred after the fifth day of treatment.

DISCUSSION

Earlier studies in patients who had nontropical sprue (celiac disease) have shown that gluten contains a peptide which damages intestinal mucosa (13). This peptide consists of 6 to 8 amino acids (14), and is no longer damaging after complete acid hydrolysis or deamination (13). Peptic and tryptic digests of gluten, including their dialysates or ultrafiltrates, produce steatorrhea in patients with celiac disease but not in normal subjects (15, 16). These digests of gluten lose their toxicity when further digested by fresh extracts of hog intestinal mucosa or crude papain (14, 16).

Previous studies from this laboratory have shown no depression of intestinal lactase activity by dietary gluten.[8] Lactase activity (7)[9] and monosaccharide absorption (8, 9, 17) are, however, significantly influenced by neomycin alone. Neomycin-induced abnormalities in intestinal fat transport may be related to the presence of gluten in the diet. Previous studies[10] showed a striking accumulation of fat in rat jejunal mucosal cells following neomycin administration to rats fed a gluten-containing diet. There was no apparent increase in animals fed gluten or given neomycin but fed a gluten-free diet. Recently, the observation of neomycin-induced lipid accumulation within absorptive cells has been confirmed in both humans[11,12] and rats.[13] Although these reports do not contain dietary information, it is probable that the subjects ate normal amounts of gluten-containing foods.

In the present study, experiment 1 showed that mean dipeptidase activity was significantly decreased in the presence of neomycin plus gluten. The dipeptidase activity in the control group suggests that the concentration of gluten used did not depress enzyme activity. In contrast, experiment 2 showed that neomycin did not reduce mean dipeptidase activity in the absence of dietary gluten. A gluten-free diet, however, did not protect against neomycin-induced diarrhea.

The rats used in experiment 1 were older adults and therefore weighed more than those used in experiment 2. Since experiments 1 and 2 were independent, it was not deemed necessary to use rats of identical age. However, the mean dipeptidase activity of the older control rats fed 10% gluten plus 8% casein in experiment 1 did not differ significantly from that of the younger control rats fed 18% casein (gluten-free) in experiment 2. This is consistent with the concept that neither age nor diet influenced the enzyme activities of the control groups. The studies of Lindberg (18) suggest that intestinal dipeptidase activity remains unchanged in adult rats. Studies of some other rat intestinal digestive enzymes, disaccharidases, show that activities remain relatively constant in adults (19).

The observed depression of dipeptidase activity suggests that increased fecal nitrogen during neomycin administration (1–3) is a consequence, at least in part, of impaired intestinal digestion of protein. Why this was observed in only rats fed gluten is unknown. Possibly, neomycin interferes with the digestion of a toxic peptide in gluten.

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Effect of Prolonged Alcohol Consumption in Rats on Pancreatic Protein Synthesis [1,2]

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ABSTRACT The objective of this investigation was to determine the effect of prolonged alcohol consumption in rats on the biosynthesis of pancreatic proteins, including trypsin and ribonuclease, employing both in vivo and in vitro techniques. Rats of the Sprague-Dawley strain initially weighing between 250 and 270 g were fed an adequate regular diet and maintained on 20% ethanol as the sole drinking fluid during a 48-week period of observations. Control rats received the same diet with water ad libitum. Pancreatic protein synthesis in vivo and in vitro was studied in these groups at 10-week intervals. The in vivo protein synthesis was evaluated by the incorporation of intravenously injected DL-leucine-1-^{14}C into the pancreatic tissue protein 45 minutes after the injection. For the studies on the in vitro synthesis of protein, slices of the pancreas were incubated for 4 hours with an amino acid mixture containing DL-leucine-1-^{14}C and an ATP-generating system. The protein synthesis was determined by: 1) the incorporation of radioactive leucine into pancreatic protein; 2) by tryptic activity; and 3) ribonuclease activity. Both the in vivo and in vitro experiments demonstrated that there is a decrease in protein synthesis including that of trypsin and ribonuclease by the pancreas of animals maintained on 20% ethanol. Food intake determinations indicated that the foregoing results were due to an effect of ethanol per se on pancreatic tissue, rather than to any major alteration of caloric or protein intake.

Chronic pancreatitis in man is commonly associated with chronic alcoholism (1, 2). Also, Menguy et al. (3) noted the development of pancreatic necrosis in dogs whose ducts were ligated immediately after feeding. They also noted that the perfusion of the duodenum with alcohol markedly elevated the pancreatic intraductal pressure. Since the main function of the pancreatic acinar cell is enzyme synthesis, it was of interest to determine the effect of ethanol (alcohol and ethanol are used synonymously in this paper) feeding on pancreatic protein synthesis. Investigations carried out in this laboratory (4) indicated that ductal ligation consistently depressed protein synthesis as measured by the incorporation of labeled DL-leucine injected into the femoral vein of the rat. Acute alcoholism, alcoholism combined with starvation, and protein-deficient diets, however, were found to have no specific effect on protein synthesis. The present paper describes the effect of prolonged treatment of rats with ethanol on the in vivo and in vitro synthesis of pancreatic protein.

EXPERIMENTAL

Rats of the Sprague-Dawley strain (initial weight range between 250 and 270 g) were divided into two groups and housed two to a cage. One group had access to water and served as controls. The other group of animals was given 10% (v/v) ethanol as the sole drinking fluid. After a 2-week period of adjustment, the alcohol concentration was increased to 20% (v/v) and was kept at this level throughout the experimental study. The diet for both of the groups consisted of laboratory ration,[3] ad libitum.

To stimulate the secretion of digestive enzymes and zymogens by the pancreas, each rat was given an intraperitoneal injection of 50 μg of carbachol [4] in 0.2 ml of

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[2] Supported by National Institutes of Health Grants nos. AM-11096 and MH-13177 and a grant from the Michigan Heart Association.

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

[4] Obtained from Merck, Sharp and Dohme, West Point, Pennsylvania.

saline 2 hours and 1 hour, respectively, prior to the start of the experiment (5, 6). For the in vivo studies, the animals were then injected in the femoral vein with DL-leucine-1-^{14}C [5] in the amount of 4 μCi/100 g body weight. After exactly 45 minutes, laparotomy was performed under light ether anesthesia. The pancreas was rapidly removed, washed in cold distilled water, weighed and then suspended in 10 volumes of 0.25 M sucrose solution and homogenized in the cold room using a Kontes glass homogenizer. The homogenate was treated with an equal volume of 10% trichloroacetic acid (TCA) and the mixture was centrifuged to separate the protein precipitate. The precipitate was washed twice with 5% TCA, followed by washing ten times with distilled water, twice with alcohol, twice with alcohol–ether mixture (3:1) and four times with ether. The precipitate was next dried to a constant weight in a vacuum oven. A weighed amount of the dried sample was put into a vial and 2 ml of hyamine hydroxide [6] were added to it. The vial was kept in a water bath at 56° for 16 hours. Fifteen milliliters of liquid scintillator were then added to the vial and the radioactivity of the sample measured on a Packard Tri-carb liquid scintillation counter, using the internal standard method of counting.

For the in vitro experiments, the rats were killed 1 hour after the second injection of carbachol. The pancreases were quickly removed, trimmed free of fat and connective tissue, and cut into pieces weighing between 90 and 100 mg. Oxygen uptake by the pancreas slices was determined by the conventional Warburg technique (7). Two pieces of pancreas were accurately weighed and introduced into the Warburg vessel containing 0.5 ml of amino acid mixture (mix. 1),[7] 0.5 ml of glucose mixture (mix. 2) [8] and 3 ml of Krebs-Ringer phosphate buffer in which the concentration of potassium and magnesium was doubled. The center well contained 0.3 ml of 5% potassium hydroxide. The flasks were incubated for 4 hours at 30°. After the incubation period, the vessels were quickly chilled, the potassium hydroxide was removed from the center well and 2.5 ml of IN HCl was added to the reaction mixture. The pieces of pancreas were homogenized in the acidified medium and the final volume of the homogenate was made up to 10 ml with distilled water. Five milliliters of the homogenate were mixed with 5 ml of 10% TCA and treated as described above for the radioactive measurements.

The determination of tryptic activity was by a modification of the method previously described (8). To 1 ml of the homogenate was added 0.25 ml of a solution containing 1 M calcium chloride and 2 M tris buffer which, with the hydrochloric acid from the homogenate yielded a pH of 8.1. Five micrograms of lyophilized crystalline trypsin were added, and the mixture was kept at room temperature for 16 hours and was then diluted to 50 ml with water. The tryptic activity in the diluted solution was determined by its action on TAME (*p*-tosyl-arginyl methyl ester). The methanol released by the reaction was determined fluorometrically (9).

The ribonuclease activity was determined by using the method of Kalnitsky et al. (10), where the hydrolysis of RNA at pH 5.0 is determined by measuring the amount of acid soluble oligonucleotide liberated. The absorbency was read at 260 mμ in a Beckman DB spectrophotometer.

RESULTS

Table 1 presents the weight gain of rats, both normal and alcohol-treated. The normal rats increased in body weight rapidly in the beginning, and then gradually. The ethanol-treated animals gained weight more rapidly in the initial stages than the normal controls. After a period of 30 weeks of alcohol feeding, however, the body weight began to decline. The rapid increase in weight during the early stages of alcohol treatment may be due to the in-

[5] DL-Leucine-1-^{14}C (0.1 mCi/ml; 0.29 mg) was obtained from Atomic Accessories, Inc., Valley Stream, New York.

[6] Hyamine hydroxide obtained from the Packard Corporation.

[7] Mixture 1: (in mg) The amino acid mixture was prepared by dissolving together alanine, 5; aspartic acid, 5; cystine, 1; glutamic acid, 10; glycine, 5; proline, 7; serine, 6; arginine, 10; histidine, 9; isoleucine, 5; leucine, 7; lysine, 10; methionine, 3; phenylalanine, 5; threonine, 10; tryptophan, 3; tyrosine, 1; valine, 6; and 2.5 ml of labeled leucine-1-^{14}C. The pH was adjusted to 7.4 and the solution diluted to 10 ml.

[8] Mixture 2: The glucose mixture was prepared by dissolving together glucose, 200 mg; fumarate potassium, 100 mg; asparagine, 25 ml; and penicillin, 2000 units; and adjusting the pH of the mixture to 7.4 and diluting to 10 ml.

TABLE 1

Body weights [1] of control and alcohol-treated rats

No. of weeks	Controls	Alcohol-treated (20% v/v)
0	260(250–270)	260(250–270)
10	338(314–350)	366(340–385)
20	398(368–415)	444(428–459)
30	438(426–461)	490(479–506)
40	470(454–488)	466(445–484)
46	480(474–496)	446(438–462)

[1] Values given are group averages (in grams) and ranges initially for 100 rats in each group. Subsequent values are averages of at least 8 animals/group.

crease in appetite and hence, excess calorie intake. In later stages, liver damage probably occurred with a resulting decrease in food intake. This may be the reason for a decrease in weight after the 30-week period of alcohol treatment.

In preliminary experiments, the maximum incorporation of injected ^{14}C leucine into the TCA precipitated protein of the pancreas was reached between 35 and 40 minutes after the injection of labeled amino acid. The specific activity (cpm/mg protein) remained more or less steady for the next 15 minutes after which it gradually began to fall. In the present in vivo study, the pancreas was, therefore, removed exactly 45 minutes after the injection of radioactive leucine.

Table 2 shows the relationship of ethanol feeding (number of weeks alcohol fed) to the rate of pancreatic protein synthesis as measured by the rate of incorporation of labeled leucine into the TCA-precipitated proteins of the pancreatic tissue. As is seen, the specific activity of the pancreatic protein showed a gradual decrease with the number of weeks of alcohol feeding. In 40 weeks, there was approximately 30% decrease and in 46 weeks, the decrease was almost 50%. The results of the control groups studied at zero, 20, 40, and 48 weeks of the experiment indicate that there is no significant change in the incorporation of labeled leucine into the pancreatic protein with respect to age of the animals.

Tables 3 and 4 show the results of the in vitro studies. The respiration rate of the pancreatic tissue as measured by the oxygen uptake began to decrease with the number of weeks of alcohol treatment. The decrease in respiration rate paralleled the decrease in specific activity of the pancreatic protein (table 3), the tryptic activity and the ribonuclease activity (table 4). The preformed tryptic activity present in the tissue (figures in parentheses, table 4) decreased with the number of weeks of ethanol feeding but the preformed ribonuclease activity remained essentially unchanged.

These results taken together indicate that chronic ethanol treatment in the rat has an inhibitory effect on protein synthesis, including that of trypsin and ribonuclease, of the pancreas.

DISCUSSION

Reports from several groups of workers provide evidence which indicates that alcohol-treated human and animal subjects have metabolic patterns which distinguish them from nonalcoholics (11). Siegel et al.[9] presented evidence indicating imbalances of plasma amino acids in alcoholics, and group distinguishing alterations of the plasma amino acid patterns following

[9] Siegel, F. L., M. K. Roach and W. B. Deville 1963 Plasma amino acid patterns in alcoholics: Ethanol induced modification. Federation Proc., 22: 680 (abstract).

TABLE 2

Effect of ethanol on pancreatic protein synthesis — in vivo studies

No. of exp.		No. of weeks on ethanol	Specific activity [1]	
Control	Ethanol-treated		Control	Ethanol-treated
8	0	0(normals)	1628 ± 192	—
0	8	10	—	1485 ± 202
5	8	20	1530 ± 158	1568 ± 194
0	8	30	—	1418 ± 159
6	8	40	1598 ± 174	1054 ± 188
6	6	48	1684 ± 218	733 ± 156

[1] Counts per minute per milligram pancreatic protein 45 minutes after the administration of DL-leucine 1-^{14}C (0.1 mCi/ml; 0.29 mg). Values are group averages with standard deviations. Duplicate runs were made on each tissue and agreed within ± 15%.

TABLE 3

Effect of ethanol on pancreatic protein synthesis — in vitro studies

No. of Exp.	No. of weeks on ethanol	Specific activity [1]	Oxygen uptake [2]
20	0 (normals)	3245 ± 425	492 ± 64
8	10	3148 ± 455	568 ± 58
8	20	2522 ± 526	481 ± 70
8	30	2246 ± 115	462 ± 61
8	40	1148 ± 214	212 ± 46
8	46	1042 ± 162	254 ± 48

[1] Counts per minute per milligram pancreatic tissue protein 4 hours after incubation with amino acid mixture containing DL-leucine 1-^{14}C.
[2] Microliters of oxygen per gram of tissue per hour. Values are group averages with standard deviations. Triplicate runs were made on each tissue and agreed within ± 15%.

TABLE 4

Effect of ethanol on pancreatic protein synthesis — in vitro studies

No. of Exp.	No. of weeks on ethanol	Trypsin [1]	Ribonuclease [1]
		mg/g	*μg/g*
20	0 (normals)	4.15 ± 0.28 (1.44 ± 0.22) [2]	433 ± 53 (162 ± 34) [2]
8	10	3.85 ± 0.30 (1.57 ± 0.18)	480 ± 66 (130 ± 25)
8	20	3.65 ± 0.35 (1.40 ± 0.16)	414 ± 54 (158 ± 31)
8	30	3.48 ± 0.22 (1.25 ± 0.16)	366 ± 56 (168 ± 28)
8	40	2.31 ± 0.28 (0.98 ± 0.15)	188 ± 26 (160 ± 32)
8	46	2.30 ± 0.26 (0.88 ± 0.16)	200 ± 34 (148 ± 20)

[1] Increase in tissue enzyme activity after 4-hour incubation period. Values are group averages with standard deviations. Triplicate runs were made on each tissue and agreed within ± 15%.
[2] Values in parentheses indicate the activities of enzymes formed (present) in the tissue incubated in the medium not containing amino acid mixture.

acute ethanol ingestion by alcoholics and controls. More recently, these authors (12) have shown that alcoholics displayed elevated blood levels of glutamic acid and lowered blood levels of methionine, leucine, valine, and isoleucine, in the blood as compared with controls (nonalcoholics). According to these authors, the elevation of the level of glutamic acid may be due to a block in its conversion to glutamine, whereas the lowered levels of other amino acids may be due to their utilization as transamination precursors of glutamine. The change in amino acid composition could affect the pattern of protein synthesis of the tissues including the pancreas. The in vivo experimental data presented (table 2) indicate a decrease in protein synthesis as measured by incorporation of labeled leucine into TCA-precipitated protein with prolonged alcohol feeding to rats. If this is true then in vitro synthesis of protein by pancreatic slices from alcoholic rats in the presence of a complete mixture of amino acids should show no significant change as compared with the pancreatic slices from the control (nonalcohol) group. The in vitro studies, however, show a decrease in protein synthesis similar to that observed in experiments in vivo. A decrease in the oxidation rate by the pancreatic tissue was observed with prolonged alcohol feeding. It appears, therefore, that alcohol consumption decreases the respiratory rate of the pancreatic tissue just as it does of liver tissue (13, 14). The oxidation coupled with

phosphorylation serves to meet the energy requirements for protein synthesis. A decrease in oxygen uptake would lower the rate of synthesis of ATP and, in turn, decrease protein synthesis and this, indeed, is observed in the in vitro experiments reported here.

A possible mechanism for fatty infiltration of the liver seen in humans and animals after prolonged bouts of heavy drinking can be proposed on the basis of the results of these experiments. Since there is a considerable decrease in pancreatic protein synthesis, including trypsin and ribonuclease, after prolonged alcohol treatment, the digestion and, hence, absorption of dietary proteins may be impaired leading to a deficiency of choline and other lipotropic factors (15). This, in turn, could lead to the development of fatty livers as was observed. Of course, other explanations are possible (16).

The relationship between prolonged ethanol intake and pancreatitis in man is a puzzling one. Ethanol may somehow affect the permeability of the cell membrane resulting in leakage of zymogen granules from the pancreatic cell. In the normal process, zymogens are secreted according to the needs for digestion, but after ethanol intake, excessive amounts may be secreted. Some of the active proteolytic enzymes could conceivably reenter pancreatic tissue and activate the zymogens being formed intracellularly. The pancreatic tissue from animals treated with ethanol, however, even after 48 weeks, had no measurable free tryptic activity, suggesting that the trypsinogen was not intracellularly activated. It is possible, of course, that pancreatic malfunction is caused not by alcohol alone, but by substances other than alcohol present in alcoholic beverages. Further work is needed to investigate this possibility.

The question logically arises as to the effect of alcohol on the daily food and protein intake and its possible relation to the data presented in this paper, specifically, the results on protein synthesis by the pancreas of the ethanol-treated rats. The food intakes of the control and ethanol-treated groups were determined at intervals during and at the end of the 40-week period, just prior to the determination of pancreatic protein synthesis, as described. The group average daily food intakes of the control and ethanol-treated rats at the 40-week period of the experiment were 22.0 and 20.0 g, respectively. The commercial laboratory ration used contained 24% (minimum) crude protein. Thus, the average daily protein intakes of the control and ethanol-treated groups were 5.3 and 4.8 g, respectively, or 1.13 and 1.03 g/100 g body weight (from table 1), respectively. Indeed, a comparable group of 12 rats given 40% ethanol (v/v) for 28 weeks were found to consume an average of 0.8 g of protein per 100 g body weight/day, as compared with a 1.2 g protein intake of the control [10] rats.[11] It is difficult to attribute the decrease in in vivo protein and enzyme synthesis by the pancreas observed in the present studies to such a relatively small decrease in the protein intake of the ethanol-treated rats. Indeed, Munro (17) reported that *complete* withdrawal of protein from the diet failed to affect "RNA metabolism in the pancreas or alter the amount of RNA per cell, whereas the liver of the same animals showed marked changes in RNA metabolism." Thus, the pancreas appears to be less sensitive than the liver to a deficiency of dietary protein. Similarly, earlier experiments in this laboratory (4) demonstrated that a protein-deficient diet had no demonstrable specific effect on pancreatic protein synthesis in rats. It appears highly improbable that the relatively small decrease in dietary protein intake found in the present investigation was a complicating factor in the observed decrease in pancreatic protein synthesis in the ethanol-treated rats. Furthermore, from Lieber's current results on carefully controlled experiments (18), it even appears unlikely that a protein factor could complicate the alcohol effect on the liver. Rather, this appears to be due to ethanol per se.

It should be added, moreover, that the results obtained in the present in vitro experiments, which substantiated the in vivo studies, could not be attributed to an inadequate intake of exogenous protein. A complete amino acid mixture was present in adequate and equal amounts in the in-

[10] See footnote 9.
[11] Sardesai, V. M., and J. M. Orten, unpublished observations.

cubation medium of pancreatic tissue from both the control and ethanol-treated rats. Pancreatic tissue from the ethanol-treated animals showed a similiar decrease in protein-enzyme formation as that found in the in vivo studies. Thus, the decrease in pancreatic protein and enzyme synthesis observed in the alcohol-treated rats in this investigation appears to be due to an effect of ethanol per se.

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Maternal-Fetal Utilization of Sulfate Sulfur by the Gravid Ewe [1]

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ABSTRACT Blood-balance and maternal-fetal tissue concentration values, following single tracer doses of $^{35}SO_4$, were used to evaluate sulfate absorption and utilization in 25 yearling gravid ewes and fetuses at 3 periods of gestation. The 81% dietary sulfur excreted was almost equally divided between feces and urine. Ingested labeled sulfate, however, was rapidly absorbed, and 75% was subsequently excreted via the kidneys during 168 hours. Maternal tissue sulfur concentration was unaffected by pregnancy, but retained ^{35}S was significantly higher ($P < 0.10$) at trimesters 1 and 2 in 45-kg ewes. Absorbed ^{35}S traversed the placenta freely at the 3 trimesters, and maternal-fetal tissue concentration patterns, after 7 days, were similar. Soft tissue ^{35}S levels in third-trimester ewes peaked after 4 hours, and decreased to 168 hours. Values for most bones and placenta were highest after 24 hours, and levels decreased to 7 days. Calculated partition of ^{35}S in third-trimester ewes and products of conception indicated that after 7 days, 55.7% of that absorbed and retained was deposited in maternal tissues, and 44.3% was transferred to the total fetus (77%), the placenta (16%) and to the placental fluids (7%), respectively.

Although sulfur was early recognized as an essential nutrient, the metabolic fate of the inorganic sulfate commonly ingested with animal rations has long been a controversial subject (1, 2). The continued interest in organic sulfur compounds and interrelationships with other minerals (3–5), however, and the special contribution of dietary sulfur to nonprotein nitrogen utilization by ruminants (6–9) has resulted in convincing evidence of the economic and nutritional contribution of sulfate sulfur to animal nutrition (10–15).

Limited use has been made of ^{35}S in studies with farm animals and no information was found relative to fetal sulfur absorption or utilization in sheep. The purpose of the present study was to obtain quantitative information on sulfate absorption and utilization by the gravid ewe, and by radioisotope procedures, to measure placental permeability, fetal distribution and sulfur utilization as a function of gestation age and time in the ovine.

MATERIALS AND METHODS

Twenty-five gravid and open Louisiana native ewes were maintained with a conventional ration consisting of 50.5% ground yellow corn, 11% cottonseed meal, 10% molasses, 15% cottonseed hulls, 5% alfalfa meal, 0.5% oyster shell flour and 1% salt. Calculated chemical analysis showed it to contain 14% protein, 0.25% P, 0.4% Ca and 1.3 mg/g sulfur. At days 45, 95 and 140 of gestation, ewes were placed in metabolism units, equipped for the quantitative separate collection of feces and urine (16), and administered a single oral or intravenous tracer dose of sodium ^{35}S sulfate for blood-balance studies. The carrier-free sulfur dose was administered as 3 mCi in less than 1 ml solution/ewe. Indwelling urinary catheters were used to assure quantitative collection, to minimize fecal contamination and for measurement of subsequent kidney ^{35}S-clearance rates. At designated periods indicated in the tables, each ewe was killed for maternal-fetal tissue analyses. Seven ewes were not pregnant, and tissue values were omitted from this report. Placentas were removed intact from the 18 pregnant ewes for separate weighing and sampling of fluids, membranes and fetus for radiochemical measurements. Four ewes were killed after

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168 hours during each treatment and 2 additional third-trimester ewes were killed after 4, 24, and 48 hours, for ^{35}S kinetic comparisons with those killed at 168 hours.

Procedure for sulfur ^{35}S measurements. The analytical procedures used were adapted from those of Katz and Golden (17), amended for routine analyses of feed, feces and all animal tissues for sulfur and ^{35}S, concurrently. Samples were wet-ashed in Erlenmeyer flasks, using the perchloric acid procedure of Eldjarn and Nygaard (18). Solution was added to plasma and urine at the ratio 10:1 and to feces and animal tissues at 15:1. Samples were digested for 2 hours, evaporated to a volume of 2 to 3 ml, removed from hot plate, diluted with 10 ml deionized water and then returned to the hot plate. Since the sulfur content of most animal tissue was low, a carrier was added as 1 ml each of sodium sulfate (1%) and barium chloride (10%) to assure complete sulfate precipitation. Flasks were stoppered and shaken for 10 to 20 minutes, warmed to 50°, and the barium sulfate precipitate washed onto two preweighed glass filters with light suction (5 ml/minute), rinsed with deionized water, ethyl alcohol and acetone, and dried under a heat lamp. The glass filters facilitated ready transfer of the suspended precipitate onto tarred planchets for drying, reweighing and for subsequent ^{35}S assay and gravimetric sulfate measurements. Precipitates were maintained at a minimum weight (10 to 30 mg) to facilitate measurements of labeled sulfur, yet assure accuracy in total sulfur mass estimations.

Sulfate ^{35}S being a soft β-emitter with a maximal energy of 0.167 Mev, required consideration for self-absorption corrections to permit standardization and relative comparisons between samples of different mass. To establish the empirical relationship between sample thickness and degree of self-absorption, a standard curve was prepared by varying sample specific activity, using increments of unlabeled barium sulfate added to constant ^{35}S labeled sulfate, or by increasing ash levels of biologically bound serum and urinary ^{35}S sulfate prepared as described for tissue samples. The total mass in the 4.75 cm^2 metal cups was thereby varied from essentially zero to 50 mg, and the theoretical curve was calculated using the equation shown in figure 1, where N is the measured net activity of the sample, N_0 is the true sample activity, T is the thickness of the sample in mg/cm^2 and μ is the absorption coefficient in cm^2/mg. This calculated value of 0.303 cm^2/mg which best fitted these data, was somewhat higher than the 0.225 cm^2/mg reported by Poddar (19). For relative ^{35}S measurements in biological materials, however, reported values checked well against ^{35}S-methionine and for total methionine sulfur recovery. These differences further emphasize the need for independently determining the value for μ for each counting arrangement.

All ^{35}S was converted to the barium salt and measurements were made on sample aliquots by conventional methods, using an automatic NC gas flow, low background beta counter. Calculated counts per minute were corrected to zero mass using the projected curve described in figure 1. For convenience of data presentation and for comparative purposes, all maternal tissue values were standardized to 45 kg body weight, and balance data were employed to correct ^{35}S values to that retained in the animal body.

RESULTS AND DISCUSSION

Total sulfur in blood of ewes. The circulating blood serves as a central meta-

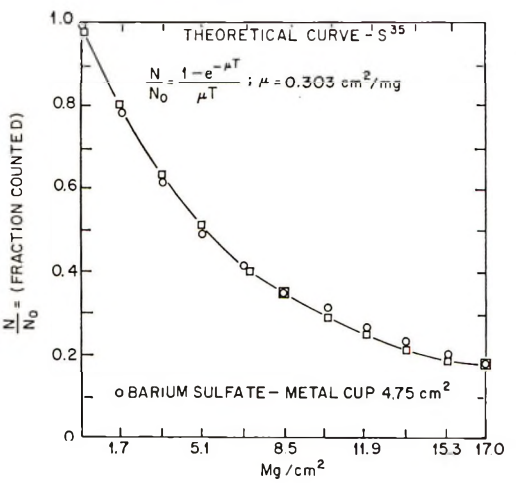

Fig. 1 Self-absorption data for ^{35}S in biological samples.

bolic pool through which all sulfur must pass in the process of absorption from the tract, transfer between soft tissue and bone, and for excretion. Total sulfur levels in blood have been shown to vary with dietary intake (2, 14, 15), animal age (9) and with various animal treatments (6, 10, 20). Serum sulfur levels have been proposed, however, as useful indexes for the sulfur status of sheep (15). Serum inorganic sulfate values for the yearling ewes in this study were within the normal range of 2 to 4 mg/100 ml (15), and averaged 2.9 ± 0.6 mg sulfur/100 ml.

Mean red blood cell and plasma ^{35}S values for gravid ewes following injection and ingestion, are shown graphically in figure 2 as a function of time after dose administration. Total blood volume was estimated as 6.5% body weight, and total red cells and plasma calculated from the hematocrits (21). The initial rapid removal pattern of the injected ^{35}S followed a characteristic disappearance curve, and reflected the combined tissue uptake, distribution and subsequent excretion effects. After 5 hours 95% of the dose had disappeared from the total circulating blood and at 24 hours 1.4% remained. Labeled sulfur was observed in the blood immediately following oral administration, increasing to 5.4% of the dose after 3 hours. Values decreased progressively with time thereafter, and disappearance rate essentially paralleled that for the intravenous radiosulfate, with less than 0.5% remaining after 4 days. Rapid absorption and movement of sulfate from the rumen has been observed by others (8, 22), and these data give supporting evidence of the dynamic nature of the element as well as the rapid movement of sulfate from the digestive tract to the blood of ruminants.

Sulfur and ^{35}S excretion. Gravid and open ewes consuming 900 ± 130 mg sulfur/day, excreted 81 ± 11% and retained 171 mg sulfur/day. The feces contained 55% and the urine 46% of the total sulfur excreted. Sulfate ^{35}S, however, was voided primarily in the urine. Seven-day cumulative excretion curves, following oral or intravenous dose administration, at the three trimesters of pregnancy are shown in figure 3. During the first 24 hours two-thirds of the 75 ± 6% urinary and one-third of the 7-day fecal (16 ± 3%) ^{35}S had been excreted, with no significant difference due either to stage of pregnancy or to method of administration. Reasons for the observed ^{35}S sulfate and die-

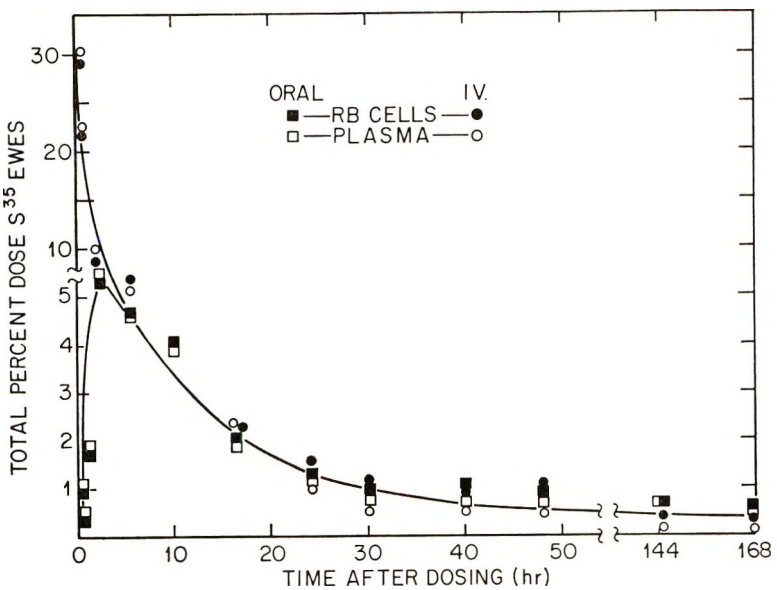

Fig. 2 Sulfur-35 in total blood of gravid ewes with time after a single oral or intravenous dose of SO_4.

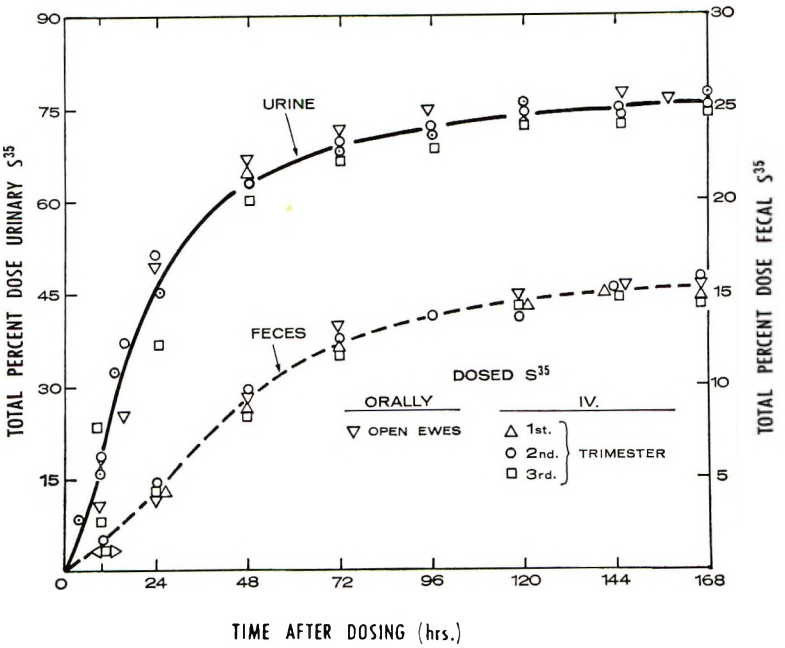

Fig. 3 Accumulative fecal and urinary excretion of orally and intravenously administered ^{35}S by open and gravid ewes at 3 trimesters of pregnancy.

TABLE 1

Gestation age effects upon total sulfur and ^{35}S in conception products of the ewe [1,2,3]

Product	Gestation age, days					
	45		95		140	
	Sulfur	^{35}S	Sulfur	^{35}S	Sulfur	^{35}S
	g	*%*	*g*	*%*	*g*	*%*
Placenta	0.450	0.90	1.68	4.1	2.55	7.1
Placenta fluids	0.022	0.03	0.05	0.7	0.17	3.0
Whole fetus	0.047	0.07	1.15	3.9	7.45	34.2
Total	0.519	1.00	2.88	8.7	10.17	44.3

[1] Total grams of sulfur and percent retained dose ^{35}S in 45-kg ewes.
[2] Values for ^{35}S include 4 fetuses at each age killed 168 hours after ^{35}S administration. Total sulfur values include analysis for 6 additional ewes and lambs at the 140-day period.
[3] About one-third of all ewes were dosed per os via stomach tube, the remainder were dosed intravenously.

tary sulfur partition differences and the high absorption of the orally administered ^{35}S, and its subsequent rapid excretion via the kidneys will require further study.

Sulfur and ^{35}S in products of conception. The effects of gestation age upon total sulfur and ^{35}S in the placenta, fluids and fetus of ewes are shown in table 1. At day 45, 86.7% of the total sulfur and 90% of the ^{35}S in the conception products were in the placenta. After 95 days, however, total sulfur and ^{35}S were nearly equally divided between the placenta and fetus. After 140 days the fetus retained 77%, the placenta 16% and the placental fluids 7% of that ^{35}S absorbed and transferred during 7 days to the products of conception.

Gestation age and maternal-fetal sulfur. Changes in tissue sulfur concentration in the ewe and her developing fetus at the three trimesters of pregnancy are shown in table 2. No significant effect of pregnancy was observed for maternal tissue,

TABLE 2

Effects of gestation age on tissue sulfur concentration [1] in ewe and fetus

Tissue	Maternal sulfur	Gestation age, days [2]		
		45	95	140
Muscle	2.5 ± 0.5 [3]	1.6	2.2	2.0
Skin	2.4 ± 0.8	1.1	2.3	2.2
Liver	3.2 ± 0.7	3.7	3.2	3.1
Kidney	2.9 ± 0.2	1.7	2.0	2.3
Adrenal	2.6 ± 0.3	—	3.0	2.8
Spleen	2.8 ± 0.1	1.9	2.9	2.9
Heart	3.6 ± 0.6	2.3	3.0	2.7
Mandible	2.4 ± 0.2	1.7	2.8	2.1
Femur shaft	2.2 ± 0.1	1.9	2.3	2.1
Femur end	3.1 ± 0.5	2.7	3.1	2.9
Rib shaft	2.5 ± 0.1	2.4	2.8	2.3
Rib end	3.2 ± 0.4	2.6	2.9	2.6
Sternum	2.8 ± 0.5	2.3	2.4	2.8
Whole fetus	— ± —	0.6	1.8	2.3

[1] Calculated as milligrams of sulfur per gram of fresh tissue.
[2] Values for ^{35}S include 4 fetuses at each age killed after 168 hours. Total sulfur values include analysis for 6 additional ewes and lambs at the 140-day period.
[3] SE of mean.

and concentration values were pooled and averaged for ease of presentation. Heart, liver, rib and femur end contained highest sulfur levels, in that order, with no marked indication of wide differences in values between maternal bone and soft tissue. Except for liver, sulfur tissue levels in the dam usually exceeded those in the fetus at all trimesters. Fetal sulfur concentration was somewhat higher at 95 days for most tissues, and at 140 days maternal-to-fetal sulfur ratios averaged 1:1.

Gestation age effects on maternal-fetal tissue ^{35}S and specific activity. Concentration of labeled sulfur in maternal soft tissues of sheep averaged higher than in bone, and after 168 hours, ^{35}S in liver, kidney and spleen were highest, followed by rib end, sternum and mandible, in that order. Fetal ^{35}S concentration values were lower at day 45 of gestation, but levels in liver and most bone exceeded those of the dam at trimesters 2 and 3. These relationships are reflected in the tissue specific activity values shown in table 3. The percentage dose of ^{35}S retained per kilogram sulfur, after 7 days, increased significantly in maternal tissues between trimesters 1 and 2 ($P < 0.01$), with little change during the final period. Maternal values were generally higher for soft tissues than bone; however, except for liver, mean differences were not great. Fetal tissue specific activity values, reflecting blood supply, metabolic activity and functional development, averaged higher during trimester 2 with greater levels in liver, rib ends and trabecular bone. The observed lack of wide differences among tissues at these periods may have been due in part at least to the dynamic nature of sulfur, and after 168 hours ^{35}S equilibrium between tissues had occurred. Comparative tissue concentrations suggested little special storage capacity for this element.

Partition of ^{35}S in total maternal and fetal organs. The calculated percent retained ^{35}S dose in total liver, kidney, spleen and heart of the ewe and fetus at the three trimesters of pregnancy are shown

TABLE 3

Gestation age effects upon maternal and fetal tissue specific activity [1] in sheep

Tissue	Gestation age, days					
	45		95		140	
	Dam	Fetus	Dam	Fetus	Dam	Fetus
Muscle	1.4	1.6	2.3	2.6	2.1	2.1
Liver	4.1	1.0	5.7	7.0	5.6	7.0
Kidney	2.5	1.6	5.1	3.4	5.1	3.0
Spleen	2.2	1.5	3.6	2.5	4.1	2.7
Heart	1.0	0.6	2.3	2.0	2.4	2.1
Skin	2.5	3.2	4.2	3.8	3.8	3.9
Femur shaft	1.1	2.2	2.3	4.2	2.3	4.0
Femur end	1.0	2.4	2.4	2.8	2.7	4.7
Rib shaft	1.3	1.3	3.4	5.5	4.0	5.5
Rib end	1.8	2.4	3.8	10.4	4.2	9.9
Sternum	1.9	1.8	3.2	3.4	4.0	5.0

[1] Specific activity calculated as percentage retained ^{35}S dose per kilogram S, corrected to 45-kg ewe.

in table 4. Trends were in the same direction as those for tissue sulfur concentration values, and magnitude of differences due to stage of pregnancy was indicated. Maternal ^{35}S increased significantly in all organs between trimesters 1 and 2 ($P < 0.01$), and liver averaged highest for both dam and fetus. Total values for spleen and kidney were similar, and the higher ^{35}S in heart may have been associated with the higher protein content.

Rate of ^{35}S accumulation in selected maternal and fetal tissue. The data in table 5 show the ^{35}S concentration in selected tissues of third-trimester ewes and fetuses killed at intervals to 168 hours after ^{35}S dosing. Peak ^{35}S concentration had already occurred in most maternal soft tissues at 4 hours after dose administration. Brain, bile and spleen were the only exceptions. Initial ^{35}S concentrations were highest in liver, kidney, adrenal, skin and aorta, in that order. Levels decreased rapidly and progressively at 24 and 48 hours, and were lowest after 168 hours. Maternal bone ^{35}S concentrations, however, averaged highest after 24 hours, with the exception of sternum where blood supply and exchange rates were probably greater. Concentration levels decreased rapidly to 168 hours, when values for trabecular bone reached one-fourth and that for cortical bone one-half peak levels. Ratio of tissue to blood ^{35}S concentration at the same time inter-

TABLE 4

Total organ ^{35}S in ewe and fetus [1] at 3 trimesters of pregnancy

Trimester (days)	Total ^{35}S retained							
	Liver		Kidney		Spleen		Heart	
	Dam	Fetus	Dam	Fetus	Dam	Fetus	Dam	Fetus
1(45)	9.4	0.04	0.5	—	0.6	—	0.8	—
2(95)	13.0	0.80	1.1	0.02	1.2	0.007	2.1	0.03
3(140)	12.9	2.10	1.0	0.07	1.2	0.020	1.8	0.15

[1] Calculated as percent retained ^{35}S dose in total organ, standardized to 45-kg ewes.

TABLE 5

Effects of time upon ^{35}S concentration in selected maternal and fetal tissue of third-trimester ewes [1]

Tissue	Time after dosing, hours							
	4		24		48		168	
	Dam	Fetus	Dam	Fetus	Dam	Fetus	Dam	Fetus
Blood	27.0	1.3	5.0	3.5	2.4	—	1.5	0.5
Muscle	14.5	0.9	9.7	2.1	7.6	5.3	5.2	5.2
Pituitary	17.0	2.0	14.2	10.6	10.2	10.2	7.6	11.5
Brain	8.1	0.1	15.1	0.7	13.8	10.0	7.6	12.3
Liver	164.2	2.2	89.3	8.0	44.0	11.0	18.3	21.6
Bile	21.0	0.8	28.0	—	18.2	6.3	4.4	3.8
Kidney	114.2	1.8	86.3	3.0	48.2	5.1	14.8	7.1
Spleen	44.6	1.4	47.2	4.1	22.2	5.2	11.6	5.9
Adrenal	72.8	0.6	63.1	2.1	24.2	5.3	14.1	5.2
Pancreas	33.4	0.9	20.1	2.0	15.2	4.5	9.5	6.3
Heart	55.2	0.7	22.2	2.2	13.5	3.5	8.6	5.8
Aorta	59.1	0.8	55.2	—	25.3	3.5	9.5	6.3
Skin	68.3	1.1	41.3	2.2	22.4	7.0	9.6	6.8
Mandible	18.1	0.5	26.3	3.5	14.1	9.2	8.4	10.8
Femur shaft	9.2	0.5	9.6	6.7	9.0	7.1	5.0	8.2
Femur end	36.1	0.9	44.0	14.1	14.0	14.1	8.0	13.1
Rib shaft	18.0	0.8	21.0	7.0	12.1	8.9	10.0	13.2
Rib end	42.2	2.2	48.1	9.1	19.2	18.3	13.2	21.1
Sternum	55.0	2.1	52.2	13.1	14.0	13.5	11.0	14.0

[1] Calculated as percent dose $^{35}S \times 10^{-4}$ per gram in 45-kg ewes.

vals would suggest that some delay in passage was involved for most tissues, and levels were highest in organs and metabolically active areas of bone. However, the biological half-life increased progressively with time-periods for most tissues to 168 hours after dose administration.

Concentration of labeled sulfur in fetal tissues reflected rate of placental transfer as well as the avidity of selected tissue for ^{35}S, and observed peak time periods would Highest concentration values for most or- suggest selective tissue rate of uptake. gans and bone were at 7 days, while for blood, muscle, bile, adrenal, skin and femur-end peak concentration had occurred before the 48-hour interval after dosing. Data, therefore, suggest that ^{35}S transferred to the fetus was removed from the circulating blood for excretion or accretion, or both, by competing processes similar to those observed for the dam, and although rate was greater, fetal tissue distribution paralleled maternal patterns.

The magnitude of these tissue ^{35}S differences in quantity and rate is illustrated in table 6 for total organs of third-trimester dams and fetuses that were killed at 4, 24, 48 and 168 hours after intravenous dose administration. Maternal liver, placenta, heart, kidney, and spleen contained nearly one-fifth of the total ^{35}S dose at 4 hours, and values decreased in all organs, except total placenta, where level increased slightly to 24 hours before its progressive decline. Liver ^{35}S decreased from 11.5 to 6.3% during the first 24 hours, and subsequently to 1.29% of the initial dose at 7 days. For direct comparison of concentration changes with time, organ values were not corrected to retained ^{35}S. Values for labeled sulfur in total fetal organs at day 140 reflect the rate of placental transfer and subsequent tissue uptake by the fetus. Peak levels were not observed before 168 hours, and liver values showed a fourfold increase between 4 and 24 hours. Level at 7 days was 10 times that after 4 hours; however, the most rapid rate was between 4 and 48 hours, when ^{35}S values were increased 7 times.

Partition of labeled sulfur by the gravid ewe. Balance studies with 25 gravid ewes showed a net retention of $9 \pm 2\%$ of administered ^{35}S dose after 168 hours. The rapid appearance into the circulating blood, and subsequent urinary excretion following oral dose administration would suggest (15, 22) initial ^{35}S absorption to be in the sulfate form. Delayed tissue equilibrium and the observed variable rates of movement of the absorbed ^{35}S into and from the different maternal and fetal organs, however, demonstrate that at least a part of the labeled element was being incorporated into organic substances. The partition of absorbed minerals by the gravid ewe may be explained on the basis of metabolic rate, and observed differences in ^{35}S turnover rate in fetal and maternal tissue (table 3) support these relationships. Radiochemical analyses show the total placental complex to contain only 1% of the absorbed ^{35}S dose after 45 days. In the final trimester, however, 44.3% of that ^{35}S retained (table 1) after 7 days was deposited in the placenta (16), fluids (7) and fetus (77), respectively. By difference then the remaining 55.7% was retained by maternal tissues and organs. As

TABLE 6

Total ^{35}S in whole organs [1] of gravid ewes and fetuses at intervals after dose administration

Organ	Hours after dosing							
	4		24		48		168	
	Dam	Fetus	Dam	Fetus	Dam	Fetus	Dam	Fetus
Liver	11.5	0.021	6.3	0.080	3.1	0.150	1.29	0.210
Kidney	0.8	0.002	0.6	0.003	0.3	0.005	0.10	0.007
Spleen	0.5	0.001	0.5	0.002	0.2	0.003	0.12	0.003
Heart	0.9	0.001	0.5	0.003	0.3	0.005	0.18	0.008
Placenta	5.4	—	5.8	—	2.6	—	0.91	—
Placental fluids	0.04	—	0.3	—	3.6	—	1.22	—

[1] Calculated as percent dose per gram $\times 10^{-4}$ fresh weight $\times$ organ weight, corrected to 45-kg ewe.

more information is made available on factors influencing maternal accretion and the metabolic behavior differences between inorganic and organic sulfur, these coefficients of utilization may provide useful guides for estimating maternal dietary requirements during the reproduction cycle in the ewe.

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In vitro Activity of Ribosomes and RNA Content of Skeletal Muscle in Young Rats Fed Adequate or Low Protein [1,2]

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ABSTRACT Young rats (89 g) were fed an adequate (18% casein) or low (3% casein) protein diet for 30 days. The content of RNA in thigh muscle, the sedimentation of ribosomes in sucrose gradients, and the synthetic activity of ribosomes in a cell-free system were studied at intervals during the experiment. The concentration of thigh-muscle RNA decreased during growth in the well-nourished rats, but the absolute amount of RNA increased almost threefold. The low protein diet decreased the concentration of RNA and the absolute amount in thigh muscle remained constant during the study. Ribosomes from muscle of rats fed the low protein diet were less active for protein synthesis in vitro than ribosomes from well-nourished rats. This difference was evident within the first 6 days and was maintained throughout the study. Sedimentation analysis in sucrose gradients revealed a higher proportion of lighter ribosome species in muscle of rats fed the low protein diet, but the differences in polysome profiles did not appear as marked as the consistent differences in the synthetic capacity of ribosomes obtained from muscle of the two dietary groups.

Malnutrition, due to a diet low in quality and quantity of protein, results in changes of the gross chemical composition of body tissues (1–3). In total body protein metabolism, the contribution made by muscle is important because this tissue comprises the major protein mass in the body and muscle shows a marked change of chemical composition during protein-calorie depletion in experimental animals (3–6) and man (7–9). Mendes and Waterlow (4) have reported that feeding a protein-deficient diet to rats caused a greater fall in the protein/DNA ratio of skeletal muscle than in liver.

Few studies have investigated the effects of protein intake on protein synthesis in muscle. Waterlow and Stephen (10) studied the uptake of ^{14}C-lysine into skeletal muscle of rats fed a low protein diet, and these workers concluded that the decreased uptake of lysine into muscle reflected a real decrease in the synthesis of muscle proteins. They did not observe a decrease in the amount or specific activity of the free lysine pool in muscle of the protein-deficients rats. Gaetani et al. (11) reported that the activity of the amino acid-activating enzymes in gastrocnemius muscle decreases within 30 days of feeding a protein-free diet and suggested that the amount of protein synthesis is controlled by the levels of these enzymes. Sidransky and Verney (12) found that the in vitro activity of skeletal muscle ribosomes is inhibited in rats force-fed a threonine-devoid diet. Munro and co-workers (13, 14) have shown that the mechanisms of protein synthesis in liver are rapidly sensitive to variations in amino acid supply and have proposed that the hepatic amino acid supply affects the equilibrium between polysomes, ribosomes, and their subunits (14).

We have begun an investigation of the biochemical consequences of altered protein nutrition and the factors involved in the regulation of muscle protein synthesis. The central role of the ribosomes in cytoplasmic protein synthesis is well established (15), and it has been shown that the in vitro activity of these subcellular particles in muscle is rapidly changed after giving rats growth hormone and testosterone (16). Therefore, we have fo-

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cused our attention on the ribosome. In the present study, the distribution of ribosomes in sucrose gradients, changes in the RNA content of muscle, and the in vitro protein synthesis capacity of ribosomes, obtained from skeletal muscle of young rats given adequate or low protein were examined.

MATERIALS AND METHODS

Experimental design. Male rats of the Sprague-Dawley strain were housed individually in suspended galvanized cages with wire-mesh bottoms. The experiment consisted of a total of 52 rats (mean beginning weight, 89 g). They were randomized into two groups of equal weight and fed ad libitum either an adequate or low protein diet. The composition of these diets is shown in table 1. Water was available at all times. A group of four rats was killed at the beginning of the study to serve as initial controls and groups of four rats fed either the 3 or 18% casein diets were killed after 6, 9, 10, 15, 20 and 30 days.

Procedure. Nonstarved rats were killed at 9:00 to 11:00 AM by decapitation and exsanguination. The skin was first removed from the hind leg region and the two hind limbs were removed. The muscle tissue was then quickly stripped from the bone, cleaned of excess fatty tissue, and the total weight of muscle of the paired hind limbs recorded. Following this, subsequent operations were performed at 4°.

Muscle from each rat within a group was pooled for isolation of the ribosomes and RNA analysis. The procedure which was followed for preparation of muscle ribosomes, as well as the method for sedimentation of ribosomes in sucrose gradients, have been described in detail previously (17). The cell-free system for the in vitro studies has also been described (17). The complete incubation system was carried out in a total volume of 1 ml. The pH 5 enzyme fraction used in the incubation medium was prepared (17) from the livers of well-nourished rats purchased specifically for this purpose. Incorporation of radioactivity in the in vitro system was linear with concentration of ribosomes up to 0.25 mg ribosomal RNA. All of the in vitro experiments used 50 or 100 μg of ribosomal RNA and incubations were conducted for 60 minutes at 37°, except where noted in the Results. Radioactivity of the protein was measured following the addition of 1 ml hydroxyhyamine and 10 ml of toluene containing 0.6% 2,5-diphenyloxazole (PPO) and 0.1% 1,4-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) in a Packard liquid scintillation spectrometer.

TABLE 1

Composition of low and adequate protein diets fed to rats for the study of skeletal muscle ribosome activity

	3% casein	18% casein
	% diet	
Casein	3.0	18.0
L-Methionine	0.05	0.3
Dextrin	54.17	44.0
Sucrose	27.08	22.0
Cottonseed oil [1]	10.0	10.0
Salt mix [2]	5.0	5.0
Vitamin mix [2]	0.5	0.5
Choline	0.2	0.2

[1] Wesson Oil, Wesson Oil Sales Company, Fullerton, California.
[2] Composition described previously (18).

The sedimentable RNA present in the detergent-treated postmitochondrial supernatant (17) was determined in conjunction with the preparation of ribosomes for the in vitro studies and sedimentation analysis. The postmitochondrial supernatant was diluted with an equal volume of 0.5 M sucrose prepared in the homogenizing medium (17) and then centrifuged for 4 hours at 40,000 rpm in a rotor 40 of the Spinco Model L ultracentrifuge. The pellet was washed twice with 2 ml of homogenizing medium and then frozen at − 20° until analyzed for RNA.

Pooled samples of frozen muscle, and the pellet obtained from the postmitochondrial supernatant as described above, were analyzed for RNA using yeast RNA (Sigma Type XI, purified) as the standard (18).

RESULTS

The final body weights and weights of the paired thigh muscles of rats given the low and adequate protein diets are summarized in table 2. Body weight increased threefold during the 30-day period of study in rats fed the 18% casein diet. Also, a similar weight change occurred in the total

TABLE 2

Body weights at killing, fresh weight of muscles of paired hind limbs, and RNA content of the muscle in rats fed 18% and 3% casein diets

Days of exp.	18% casein group				3% casein group			
	Body wt	Paired hind limbs			Body wt	Paired hind limbs		
		Muscle wt	RNA [1]			Muscle wt	RNA [1]	
			Total				Total	
	g	*g*	*mg/g*	*mg*	*g*	*g*	*mg/g*	*mg*
0	89 ± 4 [2]	5.44 ± 0.4	2.19	11.51	—	—	—	—
6	121 ± 8	7.77 ± 0.4	1.87	14.49	82 ± 3	5.44 ± 0.1	1.30	7.08
9	141 ± 9	9.10 ± 0.5	1.96	17.74	76 ± 5	4.93 ± 0.2	1.22	5.99
10	150 ± 7	10.01 ± 0.5	1.79	17.78	77 ± 3	4.98 ± 0.3	1.16	5.75
15	179 ± 14	13.33 ± 0.7	1.69	22.25	74 ± 3	4.98 ± 0.3	1.15	5.71
20	220 ± 4	17.03 ± 0.3	1.72	29.26	76 ± 5	4.98 ± 0.3	1.22	6.05
30	262 ± 9	19.44 ± 0.5	1.46	28.35	76 ± 5	5.35 ± 0.5	1.28	6.90

[1] Mean of duplicate analyses on a pooled muscle sample of each group.
[2] Mean ± SD of 4 rats/group.

thigh muscles of these rats. Body weight decreased from the beginning weight of 89 g to about 76 g during the first 9 days of feeding the 3% casein diet, and thereafter, little change was observed during the remaining three weeks of the experiment. Results obtained for RNA content of the thigh muscles are also summarized in table 2. For the well-nourished group the concentration of muscle RNA decreased with increase in body weight, presumably a reflection of enlargement of muscle fibers within the cell during growth (19). The absolute amount of RNA in the thigh muscle, however, increased from the initial value of 11.5 mg to about 29 mg during the course of the experiment. In comparison with these findings in well-nourished rats, the concentration of muscle RNA decreased during the first 6 days of feeding the 3% casein diet and the absolute amount of RNA in the thigh muscles was maintained at 6 to 7 mg during the following three weeks of the experiment.

The concentration of RNA which was sedimented from the detergent-treated postmitochondrial supernatant (ribosomal RNA) is summarized for the two dietary groups in table 3. The results parallel those found for total muscle RNA (table 2) as is expected if the bulk of total cellular RNA in muscle from growing rats is ribosomal RNA. This has been shown for rat liver (20, 21) and chick embryo heart (22). The fact that RNA obtained with the pellet from the postmitochondrial supernatant is only approximately 20% of total cellular RNA is most easily explained on the basis of the incomplete homogenization of muscle during standardized preparation of muscle ribosomes for the cell-free studies.

TABLE 3

Content of sedimentable RNA [1] in a postmitochondrial supernatant prepared from skeletal muscle of rats fed 18% and 3% casein diets

Day of exp.	18% casein		3% casein	
		Total muscle RNA		Total muscle RNA
	mg RNA/g [2]	%	*mg RNA/g*	%
0	0.463	21.2	—	—
6	0.412	22.0	0.286	22.0
9	0.352	18.0	0.262	21.5
10	0.409	22.9	0.273	23.6
15	0.336	19.9	0.229	20.0
20	0.299	17.4	0.242	19.9
30	0.220	15.9	0.201	16.9

[1] RNA sedimented by centrifugation of the detergent-treated postmitochondrial supernatant for 4 hours at 105,000 *g avg* in 0.25 M sucrose prepared in buffer (17) as described in Materials and Methods section.
[2] Per gram fresh muscle equivalent. Mean of a duplicate analysis on a pooled muscle sample from 4 rats in each group.

The in vitro protein synthetic capacity of ribosomes obtained from skeletal muscle of rats fed low or adequate protein is shown in table 4. Within the first 6 days of feeding the low protein diet the synthetic activity of muscle ribosomes was reduced to approximately half of the control values obtained with ribosomes from well-nourished rats. Furthermore, the reduced activity of ribosomes from rats fed

TABLE 4

Activity in a cell-free system of muscle ribosomes obtained from rats fed either an 18% or 3% casein diet

Days of exp.	Dietary group	
	18% casein	3% casein
	cpm × 10⁻²/mg RNA	
0	570 [1]	—
6	590	328(56) [2]
9	270	126(47)
10	280	128(54)
15	278	150(54)
20	366	149(41)
30	255	120(47)

[1] Ribosomes for each group were prepared from a pooled muscle sample from 4 rats. Each value is the mean of duplicate determinations.

[2] Figures in parentheses indicate in vitro activity of ribosomes obtained from rats given the 3% casein diet, expressed as a percentage of value obtained with well-nourished (18% casein) controls.

the low protein diet was maintained throughout the course of the experiment.

At day 30, a study was made of the time-course of incorporation of radioactivity by ribosomes obtained from the two dietary groups. The results, shown in figure 1, indicate that within 15 minutes the activity of ribosomes from rats fed the 3% casein diet was significantly lower than the activity of ribosomes obtained from well-nourished rats. This suggests that the stability of the ribosomes in the cell-free system was not affected by the nutritional status of the donor rats.

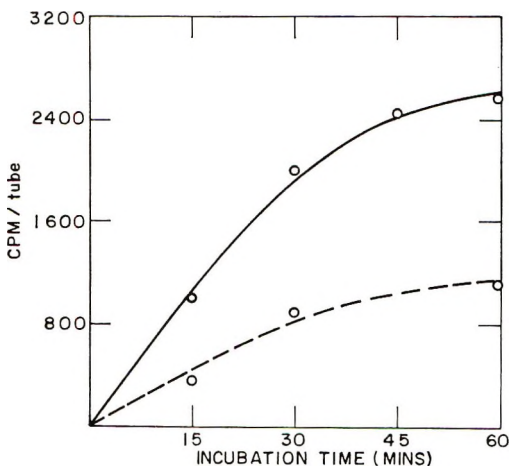

Fig. 1 Effect of protein intake on the rate of amino acid incorporation by skeletal muscle ribosomes. The incubation system is described in the Materials and Methods section and each tube contained 100 μg ribosomal RNA. Each point is the mean of duplicate determinations with ribosomes obtained from a pooled muscle sample of rats fed the 18% casein (———) or 3% casein (– – –) diet for 30 days.

Labeled amino acid incorporation in the cell-free system was measured following the mixing of equal amounts of ribosomes prepared from the two dietary groups. These results are given in table 5. The activity of the mixture was equal to the mean activity of the ribosomes prepared from the well-nourished and protein-depleted rats when incubated separately, and this suggests that, at least, the lowered in vitro capacity of ribosomes for protein synthesis from muscle of rats fed the low protein diet is not associated with a dissociable inhibitor on these ribosomes. Wool and Cavicchi (23) have conducted this type of experiment in their detailed studies of the effect of diabetes and insulin on the activity of skeletal muscle ribosomes.

The sedimentation profiles of ribosomes prepared from the skeletal muscle of the two dietary groups on days 6, 15, and 30 are shown in figure 2. The profiles obtained from the muscle of rats given the protein-deficient diet were not as well resolved as those obtained with the well-nourished rats. Furthermore, these profiles showed a greater proportion of the light ribosome (monomer and dimer) species, compared with the sedimentation profiles obtained with the muscle of well-nourished controls. The reduction in the proportion of the heavy ribosomal aggregates in the total ribosome population explains, in part, the reduced synthetic activity of the ribosomes in vitro. The differences in the profiles shown in figure 2, however, may not be great enough to entirely

TABLE 5

Effect of mixing ribosomes obtained from skeletal muscle of rats fed 18% or 3% casein diets for 20 days on amino acid incorporation in a cell-free system

Source of ribosomes	Incorporated	Change
	cpm × 10⁻²/mg RNA	%
Adequate protein (AP)	374	—
Low protein (LP)	199	−60
0.5 AP + 0.5 LP [1]	250	−33

[1] Equal amounts (50 μg) of ribosomes prepared from muscle of rats fed the adequate and low protein diets.

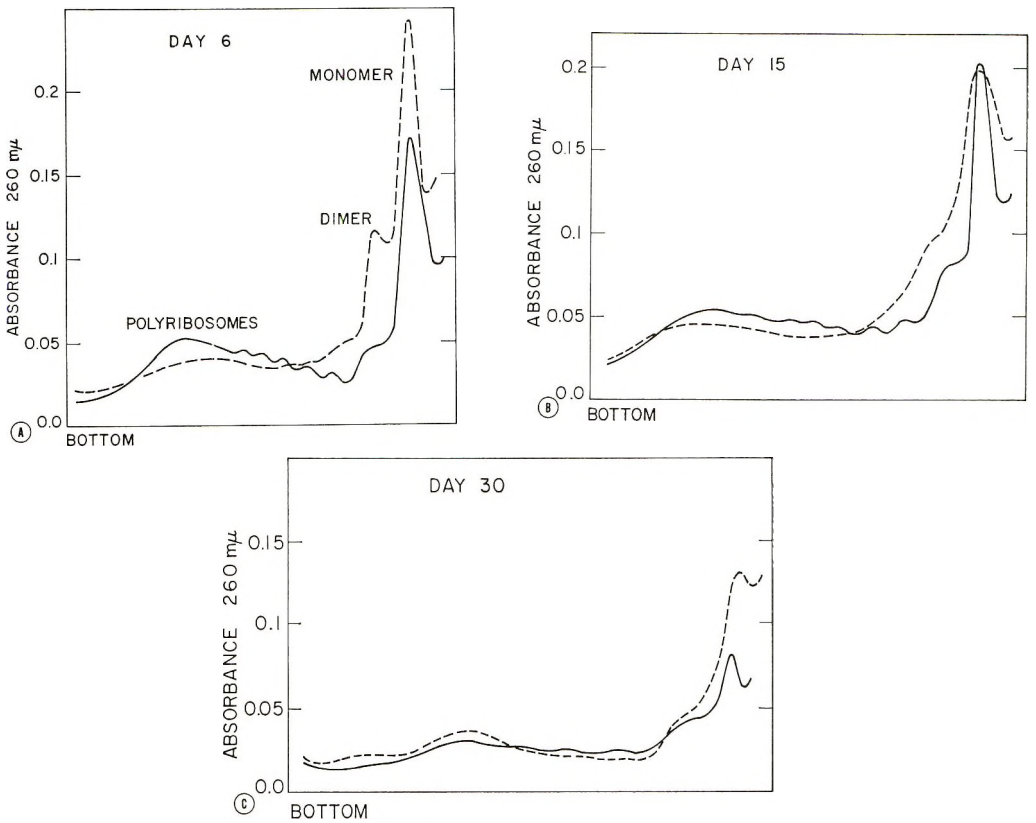

Fig. 2 Effect of protein intake on the sedimentation of rat skeletal muscle ribosomes after 6 days, 15 days, and 30 days of feeding young rats with 18% or 3% casein diets. Each curve represents the profile obtained with a detergent-treated postmitochondrial supernatant of a pool muscle sample from 4 rats and equivalent to 0.45 g fresh muscle. The gradient was withdrawn from the bottom and monitored at 260 mμ and recorded automatically using a flow cell with a 5 mm light path and a Gilford absorbance recorder. Solid line is profile obtained with rats fed the 18% casein diet and broken line with rats fed the 3% casein diet.

account for the consistent and marked differences in the in vitro synthetic activity of the ribosomes obtained from the muscle of rats fed adequate or low protein.

DISCUSSION

The results of the present experiments shed further light on the observations that in animals on a low protein diet, the muscle protein content declines (1–9), and the uptake of labeled amino acid into muscle is reduced (10). In the present study we observed that a low dietary protein intake reduced the concentration of RNA in muscle, a finding in agreement with previous studies in rats (24), dogs (25), and chicks (26). Furthermore, the protein synthetic activity of ribosomes isolated from muscle of rats given a low protein diet appears to be greatly reduced.

In well-nourished rats, the concentration of muscle RNA declined, and these results are in accordance with those of Devi et al. (19). The change observed in the present study, however, was not as great as that shown by Devi et al. (19), and this is explained by their observation that the changes in the concentration of muscle RNA, as well as DNA, are more dynamic in very young rats, whereas the present study began with rats weighing 89 g. The RNA content of muscle declined within six days of feeding a low protein diet, and thereafter the concentration did

not appear to decrease further. Although the concentration of total muscle RNA declined with increased body weight in the well-nourished rats, the absolute amount of RNA in the paired thigh muscles increased from an initial value of about 12 mg to 29 mg before the end of the 30-day study. Because the sedimentable RNA from the postmitochondrial supernatant of muscles (table 3) showed changes which paralleled total cellular RNA, it appeared that protein intake had a marked effect on the size of ribosomal RNA pool in the thigh muscles. Breuer and Florini (27) have also shown that the concentration of ribosomal RNA which was isolated from a postmitochondrial supernatant decreases in skeletal muscle with increased growth in rats.

The studies by Waterlow and Stephen (10) have shown that in rats, a low protein diet leads to a decrease in the rate of ^{14}C-lysine uptake into muscle protein and also showed that both main types of muscle protein — sarcoplasmic and fibrillar — were about equally affected. The results of the present studies offer, at least in part, an explanation as to how these changes are brought about at the subcellular level. Ribosomes obtained from the well-nourished rats (table 4) were about twice as active in vitro as ribosomes from muscle of rats fed the 3% casein diet, and this difference would be expected to result in changes in muscle protein content. However, the nature of the change in the synthetic activity of ribosomes is not known. We have previously reported (28) that feeding a protein-free diet to weanling rats for three days reduced the proportion and amount of heavy ribosome aggregates in muscle, and the present findings are in accordance with these findings. The profile changes observed in the present study do not appear to be as great as in our earlier study (28); this is probably related to the feeding of a low protein (3% casein), rather than a protein-free diet. The 3% casein diet provided sufficient protein to maintain body weight after an initial adaptation period resulting in some loss of body weight. Njaa (29) has reported that a 2 to 3% casein diet is sufficient to maintain body weight in weanling rats.

The change in the ribosome profiles in the present study is consistent with the reduced in vitro synthetic activity of the ribosomes. The changes in the polysome profiles, however, do not appear to be as marked as the consistent change in the in vitro synthetic activity of the ribosomes, and therefore, may not entirely explain the reduced ribosome activity. In this connection, a number of workers have observed changes in the rate of amino acid incorporation by hepatic ribosomes in vitro without changes in the polyribosome profiles and suggest that protein synthesis can be regulated at the level of the ribosome. Sox and Hoagland (30) have made these observations in starved and re-fed rats; Decken (31) in rats given a protein-free diet for 2 days and then given a protein-rich diet for about 14 hours; also, a number of investigators have suggested that hormones influence protein synthesis predominantly at the level of the ribosome (23, 32, 33). It seems possible, therefore, that the reduced synthetic activity of ribosomes isolated from muscle of poorly nourished rats may also be linked with regulatory mechanisms associated with the ribosomes themselves, although this study does not elucidate these mechanisms.

Waterlow and Stephen (10) suggested that the decreased uptake of labeled amino acid into muscle protein after feeding rats a low protein diet may be related to decreased insulin activity. Insulin increases amino acid incorporation into muscle protein (34), the proportion of polyribosomes in the ribosomes of muscle (28, 33), and the synthetic activity of ribosomes in a cell-free system (23, 35). Wool and co-workers (23, 35) have found that ribosomes obtained from diabetic rats show half the activity of ribosomes obtained from the insulin-treated controls and that this is due to a smaller proportion of active particles in diabetic ribosomes (36). It is interesting that in the present study the ribosome preparation obtained from skeletal muscle of rats fed the 3% casein diet was about half as active as the ribosome preparation obtained from muscle of well-nourished rats.

Results obtained in the present study provide a further understanding of the biochemical consequences of a deficient in-

take of dietary protein on skeletal muscle and demonstrate the sensitivity of the protein synthetic machinery of muscle to dietary amino acid supply. The findings, however, do not reveal the cellular mechanisms associated with the change in the activity and function of the protein synthetic machinery and, in particular, the ribosomes of this tissue.

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Chemical Composition of Kidneys from Choline-supplemented and Choline-deficient Weanling Rats [1]

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ABSTRACT A study was undertaken to determine whether the increased renal weight in choline-deficient, weanling rats is related to some specific change in the chemical composition of the kidneys. The kidneys of male weanling rats fed a choline-supplemented or a choline-deficient diet for 7 days were analyzed for moisture, total lipid, neutral lipid, phospholipid, triglyceride, cholesterol, cholesterol esters, ash, protein, RNA, and DNA. The nonlipid composition of the renal tissue from the two groups was similar. The total lipid concentrations were also similar being slightly decreased in the deficient rats. There was a drastic decrease in phospholipid concentration of the lipid from the choline-deficient kidneys and a corresponding increase in the neutral lipid. The choline, ethanolamine and phosphorus contents of the phospholipid from the two groups were similar. The moisture-free kidney tissue from a deficient rat was approximately 100 mg heavier than that from a control rat. This increase in weight was caused by an increase in total lipid, 5 mg; ash, 5 mg; protein, 82 mg; RNA, 2 mg; and DNA, 1 mg. These results suggest that the increased weight is due in part to increased cellular proliferation of the deficient kidneys. Although the kidneys of the deficient rats apparently contain increased cell numbers and possibly enlarged cells, the total phospholipid content in those kidneys was essentially the same as the controls. The increased lipid is due to an increase in triglyceride, cholesterol and cholesterol esters. The fatty acids of the triglycerides and cholesterol esters from the choline-deficient rats contained considerably more $C_{20:4}$ fatty acid.

The marked increase in organ weight that accompanies hemorrhagic degeneration of the kidneys in choline-deficient, weanling rats cannot be explained on the basis of our present knowledge of this syndrome. The striking increase in hepatic lipid content of choline-deficient animals as well as the structural role of choline in phospholipids have led a number of workers, including these authors, to proceed on the hypothesis that this increased kidney size is in some way related to alterations in renal lipid metabolism. It is in fact reasonable to assume that the increased weight may be due to some lipid component. This latter view, however, is not supported by the work of Patterson and McHenry (1), Baxter and Goodman (2), nor Fewster et al. (3). Furthermore, studies reported here give quantitative evidence that only a minor portion of the increased weight is due to lipid. We have attempted to further clarify this question by determining the relative content of a number of other constituents in the kidneys of normal and choline-deficient 7 days post-weaned rats. These included protein, ash, moisture, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). We have also studied the composition of the renal lipid fraction in detail.

EXPERIMENTAL PROCEDURE

Results reported here were based on analysis of tissue from 184 male weanling rats of the Charles River CDA strain. These rats had an average initial test weight of 56 g. One-half of the experimental animals received a choline-deficient diet. The other half received the same diet supplemented with 0.3% choline·Cl. The diets have been described in the first paper of this series (4). Essentially they were high protein, high fat (20%) diets that were limiting in methionine and vitamin B_{12}. The rats were housed in individual wire-bottom cages in an air-conditioned room and had continu-

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ous access to the experimental diets and fresh water.

On day 7 of the experiments the rats were killed and the kidneys removed, decapsulated and weighed. The tissue from 5 rats was usually pooled for analysis. Moisture was determined by drying the samples in an oven at 80° for 24 hours. Tissue samples taken for lipid analysis were homogenized in a micro-Waring Blendor containing 20 ml of 2:1 mixture of chloroform-methanol per gram of tissue. The homogenate was extracted 3 times. The chloroform-methanol extracts were washed by the method of Folch et al. (5), and the volume was reduced in a rotary evaporator under reduced pressure at 45°. The extract was transferred to a tared flask and dried under a stream of nitrogen at 45° in a water bath. Finally, the samples were dried over $CaSO_4$ under vacuum and total lipid determined by weight on a semimicro balance. The total lipid samples were separated into neutral and phospholipid fractions by the following procedure. The lipid was dissolved in 100 ml of chloroform and transferred to a flask containing 20 g of silicic acid. The flask was shaken for 20 minutes and the contents were transferred to a medium porosity fritted glass filter fitted to a vacuum flask. Under these conditions the neutral lipids remain in solution in the chloroform and the phospholipids adsorb to the silicic acid. The silicic acid was then washed with methanol to remove the phospholipids. The amount of each fraction was determined by the procedure outlined for total lipid analysis. The neutral lipid fraction was analyzed for triglyceride by the method of Van Handel and Zilversmit (6). Free and esterified cholesterol and free fatty acids were separated on a microcolumn of silicic acid (7). The quantities of cholesterol and cholesterol esters were determined by the method of Vahouny et al. (8) and the free fatty acids by the procedure of Duncombe (9). The phospholipid fraction was also tested for cholesterol. This fraction contained approximately 10% cholesterol. Appropriate adjustments were made in the phospholipid values reported based on cholesterol content. Total lipid phosphorus (10) was determined on both the neutral and phospholipid fractions. The phosphorus content of the neutral lipid fraction was insignificant. All separations were monitored by thin-layer chromatography.

Aliquots of the phospholipid fractions were hydrolyzed by refluxing in 6 N HCl for 3 hours (11). The hydrochloric acid was removed in a flash evaporator and the residue redissolved in distilled water. This solution was analyzed for choline by the method of Wall et al. (12) and for ethanolamine by the method of Axelrod et al. (13).

The fatty acid composition of cholesterol esters was determined in the following manner. The esters were hydrolyzed 4 to 5 hours in a methanol-KOH solution (0.4 N KOH) at 60°. The hydrolysis mixture was washed with hexane, acidified with concentrated H_2SO_4 and the free fatty acids extracted with hexane. The free fatty acids were methylated by refluxing in methanol-H_2SO_4 (98:2, v/v). Methyl esters of the fatty acids from triglycerides were formed by refluxing the triglycerides in a solution of 2% H_2SO_4 in methanol. The methyl esters were resolved and quantified by gas chromatography on a model DSS-Micro-Tek chromatograph fitted with a 183 cm diethylene glycol succinate (15%) column. The relative percentages of the fatty acids present were determined by summing the products of the peak heights times the width at one-half peak heights for each acid, dividing each product by this sum and multiplying by 100.

The kidney tissue taken for nucleic acid and protein analyses was homogenized in 4 ml of cold 5% trichloroacetic acid (TCA) per gram of liver. The nucleic acids were separated from the other cellular material by the method of Shibko et al. (14) with the exception that RNA and DNA were hydrolyzed together in 5% TCA. RNA was determined by the orcinol method and DNA by a diphenylamine method (15). Protein was determined gravimetrically.

RESULTS

The kidneys from the choline-deficient rats were approximately 1.6 times heavier than those from the supplemented rats and were almost without exception hemorrhagic on day 7 of the experiment. The relative concentrations of moisture, total

lipid, neutral lipid, phospholipid, ash, protein, RNA and DNA in the kidneys of choline-deficient and choline-supplemented rats are given in table 1. There was a decrease in the total lipid, RNA and DNA concentrations, and an increase in the moisture and possibly the protein concentrations of the deficient kidneys. However, all differences were relatively small. The ash concentrations were similar. The most striking difference between these 2 groups was the alteration in composition of the total lipid fraction. The renal lipids from deficient rats contained 49% neutral lipid and 50% phospholipid, whereas the same fraction from supplemented rats contained 39% neutral lipid and 61% phospholipid.

The results of our studies to determine the whole kidney composition of the two experimental groups are presented in table 2. The hemorrhagic kidneys were 530 mg heavier than the control kidneys. Moisture

TABLE 1

Moisture, lipid, ash, protein, RNA and DNA concentrations in the kidneys of choline-deficient and choline-supplemented weanling rats

	Choline-deficient rats [1]	Choline-supplemented rats
Moisture content, %	76.89 ± 0.08 [4,5]	74.71 ± 0.11
Total lipid, % of MFT [2]	11.15 ± 0.14 [5]	14.46 ± 0.11
Neutral lipid, % of TL [3]	48.68 ± 0.53 [5]	38.81 ± 2.65
Phospholipid, % of TL	49.54 ± 0.26 [5]	61.29 ± 0.71
Ash, % of MFT	5.06 ± 0.10	5.24 ± 0.20
Protein, % of MFT	64.83 ± 3.30	57.64 ± 3.52
RNA, mg/g MFT	24.20 ± 0.30 [5]	27.69 ± 0.65
DNA, mg/g MFT	14.28 ± 0.64 [5]	17.73 ± 0.39

[1] The kidneys from this group of rats were enlarged and hemorrhagic. Both groups were removed from the experimental diets on day 7.
[2] Moisture-free tissue.
[3] Total lipid.
[4] Mean ± SE, n = 3. The kidneys of 5 rats were usually pooled for analysis; however this varied somewhat in the samples used for protein analysis. RNA and DNA analyses were performed on the kidneys of individual rats. All 8 analyses reported in this table were not performed on the same groups of rats; rather this table is a composite of a number of experiments.
[5] Significantly different from supplemented rats, $P < 0.01$.

TABLE 2

Moisture, tissue, lipid, ash, protein, RNA and DNA content of whole kidneys from choline-deficient and choline-supplemented weanling rats

	Choline-deficient rats	Choline-supplemented rats	Δ
Total kidney wt/rat, mg	1340 ± 40 [1,2]	810 ± 20 [1]	530
Moisture, mg	1030	605	425
Total moisture-free kidney tissue, mg	310	205	105
Total lipid, mg	34.5	29.6	4.8
Neutral lipid, mg	16.8	11.3	
Phospholipid, mg	17.1	18.2	
Cholesterol, mg	7.5	5.5	
Cholesterol esters, mg	2.5	0.7	
Triglyceride, mg	5.7	2.4	
Free fatty acids, mg	0.3	0.3	
Lipid phosphorus, mg	0.7	0.7	
Choline, mg	1.4	1.5	
Ethanolamine, mg	0.5	0.5	
Ash, mg	15.6	10.7	4.9
Protein, mg	200	118	82
RNA, mg	7.5	5.7	1.8
DNA, mg	4.4	3.6	0.8
RNA/DNA ratio	1.7	1.6	

[1] Mean ± SE, n = 3. The kidneys of 5 rats were pooled. No other SE values were given in this table since all other values were obtained by multiplying the respective concentrations by the kidney weights or the total lipid weight.
[2] Significantly different from supplemented rats, $P < 0.01$.

accounted for 425 mg of the additional weight. All of the increased weight, however, was not moisture. The moisture-free kidneys of the deficient rats were 105 mg heavier than those of the supplemented group. The kidney weights presented in this table were taken from a group of rats that had an initial body weight on experiment of 45 g. However, the moisture-free kidneys of choline-deficient and choline-supplemented rats with an initial body weight of 63 g weighed 371 mg and 265 mg, respectively — a difference of 106 mg. Thus, the values presented in table 2 appear to be valid over this weight range (45 to 63 g).

Approximately 5 mg of the difference in moisture-free tissue could be accounted for as the increase of total lipid in the deficient kidneys. Essentially all of the increase in total lipid was related to an increase of the neutral lipid fraction (5.5 mg) and in turn the increase in neutral lipid derived from an increase in cholesterol, cholesterol esters and triglycerides. Surprisingly, the total phospholipid contents of the two groups were similar, as were the lipid phosphorus, choline and ethanolamine contents of the whole kidneys. Ash, RNA and DNA account for a small portion (8 mg) of the difference; however, 82 mg of the 105 mg was demonstrated to be protein.

One of the most striking differences, on a relative basis, noted in these experiments was the difference in cholesterol ester content between the normal and hemorrhagic kidney. The hemorrhagic kidneys contained almost 3.5 times as much cholesterol ester as the normal kidneys.

It occurred to the authors that the increased cholesterol ester and triglyceride content of the hemorrhagic kidneys might be related to increased content of certain fatty acids in the deficient rats. The results of our analysis of the fatty acids from cholesterol esters gave some support to this hypothesis. The esters from the deficient rats contained significantly more $C_{20:4}$ fatty acid than that from the control rats. These results led us to determine the fatty acid composition of the triglyceride fraction from the renal neutral lipids. The results of this analysis are presented in table 3. The relative concentration of $C_{20:4}$ fatty acid again was increased in the deficient rats. This was also true in the case of the $C_{18:2}$ fatty acid. The $C_{20:4}$ fatty acid co-chromatographed with arachidonic acid and the $C_{18:2}$ fatty acid co-chromatographed with linoleic acid.

DISCUSSION

The data demonstrate a striking similarity in the nonlipid cellular composition of normal and hemorrhagic (from choline-deficient weanling rats) renal tissue. Even the total lipid concentrations are similar, being slightly decreased in the deficient rats. The composition of the lipid fraction, however, was markedly altered. There was a relative decrease in phospholipid content of the lipid from hemorrhagic kidneys (61 to 50%) and a compensating increase in neutral lipid. Patterson and McHenry (1) reported essentially no difference in the

TABLE 3

Fatty acid composition of renal triglycerides from choline-deficient and choline-supplemented weanling rats [1]

Carbon number and unsaturation	Choline-deficient rats	Choline-supplemented rats
12:0	1.2 ± 0.1 [2]	0.9 ± 0.2
14:0	3.8 ± 0.2	3.8 ± 0.1
16:0	28.8 ± 0.2 [3]	35.8 ± 1.5
16:1	4.3 ± 0.2	4.4 ± 0.4
18:0	8.1 ± 0.2	7.5 ± 0.5
18:1	35.3 ± 1.1	37.7 ± 1.0
18:2	12.2 ± 0.2 [3]	9.2 ± 0.6
20:4	6.3 ± 0.7 [3]	3.0 ± 0.6

[1] The values presented in this table represent the relative concentrations of the fatty acids in the triglycerides.
[2] Mean ± SE, n = 3.
[3] Significantly different from supplemented rats, $P < 0.01$.

moisture, total lipid, and nitrogen concentrations and a decrease in the phospholipid concentration of normal and hemorrhagic kidneys under similar conditions. Fewster et al. (3) reported a similar decrease in phospholipid concentration of the total lipid fraction. The data reported here are in general agreement with these reports. Small differences are apparent in the DNA and RNA concentrations. Since the DNA/RNA ratios were similar in the two groups, the decrease in the concentrations of these components may be because of increased cell size in the deficient kidneys. These data (table 1) lend strong support to the argument that, with the exception of the phospholipid and neutral lipid content of the lipid fraction, little difference is apparent in the cellular composition of normal and hemorrhagic kidneys. Viewed in this light the increased weight of the hemorrhagic kidneys can be partially explained on the basis of increased renal cellular proliferation in the deficient rats. Part of the increase is because of a relatively larger increase of moisture in the hemorrhagic kidneys and possibly protein. This interpretation is supported by our observations on the renal DNA concentrations of the two groups. As previously noted, enlarged cell size may also be involved.

The results reported in table 2 clearly demonstrate that the increased weight in moisture-free hemorrhagic kidneys was due to an increase in total content of lipid, ash, protein, RNA and DNA. This was the expected result if the increased weight was because of increased cell numbers. It may, however, be an oversimplification to attribute all increased weight in the moisture-free hemorrhagic kidney tissue to increased cellular proliferation. We have noted the deposition of a protein-like material in the tubules of hemorrhagic kidneys [2] which may account for a part of the increased protein. These data (table 2) also show that, although the moisture-free kidney weight of the deficient rats was increased some 100 mg, the total phospholipid is equal to or slightly lower than that of normal kidneys. Whether the total phospholipid content of the hemorrhagic kidney is actually lower than the normal kidneys is open to some debate. Patterson and McHenry (16) in 1942 found "no significant difference between the total amounts of phospholipids in the kidneys" of choline-supplemented and choline-deficient rats. In a later study (1), however, these authors were able to demonstrate a slight decrease in the damaged kidneys (13.5 mg versus 19.5 mg). The important point to be drawn from these studies is that the total phospholipid of the hemorrhagic organ remains essentially the same but is distributed over some 100 mg of additional moisture-free tissue. The phospholipids in these cells are replaced to a large extent with neutral lipid — mainly triglyceride, cholesterol and cholesterol esters. The limiting phospholipid content of the renal tissue from deficient rats may cause the production of abnormal cells and ultimately produce acute renal shutdown. Parks and Salmon (4) noted extremely high blood urea values and decreased urine volume in rats with hemorrhagic kidneys — a condition that is indicative of renal shutdown. The increased renal cellular proliferation in deficient rats indicated in these studies also suggests the induction of a compensatory hypertrophy similar to that observed in unilateral kidney disease (17) or unilateral nephrectomy (18).

In this present study we could detect no difference in the choline, ethanolamine, or phosphorus content of the phospholipid fractions from the two groups. Thus, the decrease in phospholipid content of lipid from the kidneys of choline-deficient rats was apparently due to a decrease in all phospholipid components rather than just the choline containing phospholipids. There was, of course, a reduction in choline, ethanolamine and lipid phosphorus per unit weight of tissue or total lipid in the kidneys of choline-deficient rats. These findings for choline are in general agreement with the results of Patterson and McHenry (1). Fewster and Hall (19) have reported a decrease of 5% in the ethanolamine content of phospholipid from hemorrhagic kidneys from choline-deficient rats.

The increased level of the $C_{20:4}$ fatty acid in the renal cholesterol esters and trigly-

[2] Shields and Parks, unpublished data.

cerides from the deficient rats suggests a possible emergency transport role for cholesterol and triglyceride under conditions in which phospholipid concentrations are limited, especially choline containing phospholipids. Fewster et al. (3) have reported that the phospholipid content of arachidonic acid decreased from 28.3% in the kidneys of normal rats to 14.9% in the kidneys of choline-deficient rats. However, these workers did not find an increase in the arachidonic acid content of neutral lipid from the kidneys of deficient rats. The rats in these experiments (3) were killed after 11 to 14 days of receiving the deficient and supplemented diets.

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Vitamin A Activity of Carotenes in Corn Silage Fed to Lambs [1]

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ABSTRACT This study was conducted to compare the biopotency of the carotenes in corn silage with all-*trans*-retinyl palmitate for purposes of supporting liver vitamin A storage when fed to sheep. Fifty-six Western lambs with an average initial weight of 22.6 kg were fed a pelleted vitamin A depletion diet ad libitum for 110 days. Initial serum vitamin A levels of 28.3 μg/100 ml dropped to 20.0 μg/100 ml at the end of depletion. The lambs were allotted at random to 1 of 4 repletion diets. Lot 1 received corn silage ad libitum plus 0.16 kg/lamb/day of a protein-mineral-vitamin supplement. The total carotene content of the corn silage was 8.1 mg/kg (fresh basis, 28% dry matter). Lots 2, 3 and 4 received the modified depletion diet (90% dry matter) supplemented with 0.52, 1.55 and 4.65 mg of all-*trans*-retinyl palmitate/kg, respectively. After 70 days of repletion, serum vitamin values were 41.6, 20.4, 40.2 and 48.3 μg/100 ml for lots 1 through 4, respectively. Final liver vitamin A values (milligrams per liver), in the same order, were 12.29, 1.92, 8.35 and 15.36. The linear regression of total liver vitamin A stored with daily dietary intake of retinyl palmitate was calculated to be $\hat{Y} = 1.6038 + 1.4516X$, where $\hat{Y}$ = mg vitamin A/liver and X = mg of all-*trans*-retinyl palmitate ingested/lamb/day. Using this regression and interpolating from liver vitamin A storage when corn silage was consumed, it was established that 1 mg of corn silage carotenes was equivalent to 0.24 mg (436 IU) of all-*trans*-retinyl palmitate.

The vitamin A activities of carotenes in natural feedstuffs fed to sheep have been investigated by a number of workers. Guilbert et al. (1, 2) established the vitamin A activity of crystalline carotene dissolved in cottonseed oil and of the carotenes in alfalfa meal as compared with vitamin A in cod liver oil. Hoefer and Gallup (3) compared the vitamin A activity of alfalfa meal carotenes and of the carotenes in a carrot oil concentrate with a fish liver oil vitamin A concentrate. Myers et al. (4) used graded levels of carotene from artificially dehydrated alfalfa leaf meal and compared vitamin A activity with graded intakes of a dry vitamin A carrier containing a mixture of naturally occurring vitamin A esters.

Despite the importance of corn silage in ruminant feeding, little information is available on the vitamin A activity of the carotenes which this feed contains. Investigations with beef cattle have frequently led to contradictory conclusions. Jordan et al. (5) found that steers were depleted of their liver vitamin A stores, and exhibited deficiency symptoms, after being wintered on corn silages containing concentrations of carotene considerably in excess of supposed needs. Klosterman et al. (6), however, fed corn silages to vitamin A depleted steers, which contained lower concentrations of carotene than those used by Jordan et al. (5), and found that plasma carotene and vitamin A levels were restored to normal. These workers observed no deficiency symptoms and concluded that corn silage provided adequate vitamin A activity for steers.

Apparently no one has compared the vitamin A activity of corn silage carotenes against a carefully prepared oral vitamin A standard, and the objective of this study was to perform this task with sheep.

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MATERIAL AND METHODS

The sources of vitamin A activity used in this study were as follows.

Natural carotenes. These were supplied by corn silage which contained 8.1 mg of total carotene per kilogram of fresh weight. This level was determined by modifying AOAC (7) procedures in the following manner. Triplicate 5-g samples of fresh silage (collected 5 times during repletion) were homogenized in 100 ml of acetone using a Virtis [4] mixer operated at speeds up to 45,000 rpm. Suitable aliquots of the pigments were extracted into 5 ml of hexane and placed on Hyflo Super Cel: Sea Sorb 43 (1:1) columns (10 × 1.8 cm) followed by elution with 10% of acetone in hexane. The eluant was appropriately diluted and the optical density compared with a 100% β-carotene standard [5] in a Bausch and Lomb Spectronic 20 spectrophotometer at 440 mμ.

Retinyl palmitate. This material was obtained commercially as a vitamin A feed supplement.[6] It contained 197.2 mg of all-*trans*-retinyl palmitate per gram (by assay) plus edible tallow, gelatin, lactose and 2% of ethoxyquin. Particle surfaces were treated with sodium silico aluminate.

Feeds. The vitamin A depletion diet consisted of 40% of ground soft winter wheat, 41% of ground corn cobs, 14% of dehulled solvent soybean meal, 3.75% of cane molasses, 0.5% of trace mineral salt, 0.5% of limestone, 0.25% of yellow grease and 22 mg of irradiated yeast (supplying 4.84 μg of ergocalciferol) per kilogram. No measurable carotenes were present in this diet when assayed by AOAC (7) procedures.

The vitamin A repletion diets were prepared by substituting premixes for a portion of the wheat in the modified depletion diet (ground corn cobs increased to 45% and soybean meal and molasses decreased to 12 and 1.75%, respectively), which supplied 0.52, 1.55 or 4.65 mg of retinyl palmitate per kilogram of diet (90% dry matter). These premixes were prepared by appropriate dilutions of the vitamin A feed supplement [6] with ground soft winter wheat. Both the depletion and repletion diets were formed into 5-mm pellets using minimum steam exposure and rapid cooling.

The corn silage was produced from a double-cross dent hybrid, Michigan no. 400, planted in May 1966 and harvested on September 18 in the early-dent stage of maturity. The corn plant was chopped to produce an average particle length of 1 cm. The average dry matter content at ensiling was 29%. Nitrate levels were less than 0.2% (dry basis) according to the diphenylamine test (7). Feeding was begun after about 3.5 months of storage, and dry matter content at this time was 28%. Since dry matter was determined in a forced-draft oven at 105°, some lactic and acetic acid losses may have accompanied the loss of water during the drying of the silage. The corn silage was supplemented with 0.16 kg/lamb/day of a protein-mineral-vitamin mix which consisted of 93.7% of dehulled solvent soybean meal, 1.8% of limestone, 4.5% of trace mineral salt and 22 mg of irradiated yeast (supplying 4.84 μg of ergocalciferol) per kilogram. The dry matter supplied by the corn silage plus supplement was calculated to be isonitrogenous and isocaloric with the pelleted repletion diets containing retinyl palmitate.

Experimental design. Sixty-one crossbred lambs were purchased from a drought area in the Texas Panhandle. They were wormed twice after arrival and were vaccinated for enterotoxemia. Their average initial weight was 22.6 kg, and they were fed the vitamin A depletion diet and water ad libitum for 110 days. Blood samples were drawn from the jugular vein at the beginning of the depletion period and thereafter at days 35, 70 and 110. The serum was frozen and stored at − 25° until it was analyzed for carotene and vitamin A. Five lambs were killed due to poor performance during this period and were necropsied.

The remaining 56 lambs were allotted at random to one of four repletion treatments and were housed in 4-m × 7-m pens. Lot 1 received corn silage ad libitum plus the protein-mineral-vitamin supplement; lots 2, 3 and 4 received the pelleted

[4] Virtis No. 45, The Virtis Company, Inc., Gardiner, New York.

[5] 100% β-carotene, Catalog no. 3702, Eastman Organic Chemicals Dept., Distillation Products Industries, Rochester, New York.

[6] PGB-325S Dry Vitamin A Feed Supplement (Control no. B-10584), Distillation Products Industries, Rochester, New York. Supplied through the courtesy of S. R. Ames, Director, Biochemical Research Laboratories.

repletion diets supplying 0.52, 1.55 and 4.65 mg of retinyl palmitate per kilogram, respectively. An attempt was made to provide similar dry matter intakes for the four lots by restricting the pellets offered to lots 2, 3 and 4 to the dry weight of silage plus supplement consumed by lambs in lot 1.

Blood samples were obtained at days 35 and 70 after the start of the repletion period, and the serum was frozen and stored at − 25° until analyzed for carotene and vitamin A.

On day 71 of repletion, the lambs were slaughtered, their livers excised and weighed, and samples for vitamin A analysis were taken from the ventral lobe, starting at the umbilical fissure and excising in a caudal direction.

Analytical procedures for serum and liver. Serum vitamin A concentrations were determined using the antimony trichloride method according to Embree et al. (8). The serum was also checked for possible carotene content by measuring the optical density of the petroleum ether extract of the serum at 440 mμ using a petroleum ether blank.

Liver vitamin A concentrations were determined according to the method of Gallup and Hoefer (9) with the following modifications. Liver slices were homogenized in 45 ml of freshly prepared 4% of potassium hydroxide in 95% ethanol for 3 minutes in a Virtis mixer at 45,000 rpm. The homogenate was quantitatively diluted to 100 ml with water and after complete mixing, duplicate 10 ml aliquots were taken. These were extracted by shaking for 1 minute in 10 ml of petroleum ether (30 to 60° bp) and were then centrifuged at 2000 × *g* for 1 minute. Appropriate aliquots were taken from the petroleum ether layer and checked for the presence of carotene at 440 mμ. These aliquots were then evaporated in a vacuum oven at a temperature of 55° and a vacuum of 250 to 730 mm Hg. The dried samples were dissolved in 1 ml of chloroform, 2 drops of acetic anhydride were added and the mixture reacted with 2 ml of a 20% antimony trichloride solution. The maximum stable (for a few seconds) readings at 620 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer were recorded. The vitamin A concentrations determined by this procedure were compared with values determined by the method of Embree et al. (8) and by the method of Dugan et al. (10). The means of replicate values measured by the three methods were within a range of ± 5%.

Statistical analysis. The data were examined for statistical significance by an analysis of variance. Mean differences were compared using non-orthogonal polynomial coefficients according to Li (11).

RESULTS AND DISCUSSION

The effects of vitamin A depletion and repletion upon body weight and feed consumption are shown in tables 1 and 2. Average daily gain for lambs completing 110 days of depletion was 0.17 kg. During repletion, the attempt at restricting pellet consumption in lots 2, 3 and 4 to the dry matter intake on corn silage in lot 1 was not entirely successful because the restricted lambs began to eat their straw bedding. Thus, it was not possible to restrict pellet consumption to less than 1.75 kg/lamb/day. Average daily gains during repletion were 0.10, 0.12, 0.10 and 0.12 kg for lots 1 through 4, respectively, and were not significantly different. The final weights of the lambs in lots 3 and 4, however, were significantly ($P < 0.01$) greater than those of lambs in lots 1 and 2. While allotment to treatment was made at random and initial repletion weights did not differ significantly between lots, the tendency for lambs in lots 3 and 4 to be heavier at the beginning may have influenced final weights. It would appear that 8.1 mg of total carotene as supplied by 1 kg of corn silage or 0.52 mg of retinyl palmitate supplied by 1 kg of a complete pelleted diet were adequate to support weight gains in lambs during the latter part of the growth period (from 40 to 50 kg of body weight).

TABLE 1

Weight gain and feed consumption of lambs fed a vitamin A depletion diet for 110 days

	kg
Initial body wt (61) [1]	22.6 ± 0.52 [2]
Final body wt (56) [1]	41.2 ± 0.59
Avg daily feed [3]	1.42

[1] Numbers in parentheses indicate number of lambs.
[2] Mean ± SE.
[3] Feed expressed on 100% dry-matter basis.

TABLE 2

Weight gain and feed consumption of lambs fed rations containing carotenes or retinyl palmitate for 70 days

	Lot 1	Lot 2	Lot 3	Lot 4	
	Corn silage + supplement	Basal + 1.1 mg retinyl palmitate/day	Basal + 3.3 mg retinyl palmitate/day	Basal + 9.9 mg retinyl palmitate/day	SE
	kg	*kg*	*kg*	*kg*	
Initial body wt [1]	39.8	40.1	43.3	41.8	0.59
Final body wt [1]	47.1	48.4	50.4 [2]	50.5 [2]	0.77
Avg daily feed [1,3]	1.24 [4]	1.93	1.93	1.93	

[1] Fourteen lambs per lot.
[2] Significantly greater ($P < 0.01$) than lot 1.
[3] Feed expressed on 100% dry-matter basis.
[4] Corn silage, 1.08 kg; protein-mineral-vitamin supplement, 0.16 kg.

TABLE 3

Serum vitamin A concentration of lambs fed rations containing carotene or retinyl palmitate for 70 days

	Lot 1	Lot 2	Lot 3	Lot 4	
	Corn silage + supplement	Basal + 1.1 mg retinyl palmitate/day	Basal + 3.3 mg retinyl palmitate/day	Basal + 9.9 mg retinyl palmitate/day	SE
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*	
35 days [1]	41.3	20.8	32.8	49.7	1.81
70 days [1]	41.6 [2]	20.4	40.2 [2]	48.3 [2,3]	1.63

[1] Fourteen lambs per lot.
[2] Significantly greater ($P < 0.01$) than lot 2.
[3] Significantly greater ($P < 0.05$) than lots 1 and 3.

No serum carotenes were detected during depletion or repletion. During depletion, serum vitamin A decreased from 28.3 ± 3.9 μg/100 ml (SE) initially to 23.0 ± 3.3 at 70 days and to 20.0 ± 2.9 at 110 days. At the end of 35 days of repletion lots 1, 3 and 4 showed significant increases in serum vitamin A concentration, and these values are presented in table 3. These levels did not increase further during the remaining 35 days of repletion, except in lot 3. There was essentially no increase in lot 2, and serum vitamin A concentrations did not reach predepletion levels. At the end of repletion, lots 1, 3 and 4 were significantly ($P < 0.01$) greater than lot 2, and lot 4 was significantly ($P < 0.05$) greater than lots 1 and 3. The response of serum vitamin A concentration to treatments was linear during the repletion period at 35 and 70 days for lots 1, 2 and 4, while lot 3 showed a significant ($P < 0.005$) quadratic response at 70 days of repletion. The treatment by period within group interaction was quadratic by linear ($P < 0.05$). This was a reflection of the fact that the serum vitamin A concentration in lots 1, 2 and 4 reached a maximum at 35 days and tended to stay the same or declined to 70 days, while lot 3 continued to increase to the end of the repletion period.

The extent of liver vitamin A depletion can be judged only on the basis of liver values obtained from 4 of the 5 animals sacrificed for reasons of poor performance before the end of the depletion period. These animals were, of course, not selected at random. At necropsy, 2 of these animals exhibited signs of hemorrhagic enteritis, 1 exhibited skeletal muscle lesions suggestive of white muscle disease and 2 exhibited no detectable pathology. Comparisons of values (2.53 mg vitamin A/liver) from 3 animals killed near the start of depletion with the value (0.66 mg vita-

min A/liver) from the animal killed at 71 days would indicate that liver vitamin A depletion was occurring. No liver carotenes were detected.

The effects of repletion on liver vitamin A are shown in table 4. The lowest average liver vitamin A concentration was found in lot 2 and was significantly ($P < 0.01$) different from either lots 1, 3 or 4. Lot 3 was also significantly ($P < 0.01$) lower than lots 1 or 4. The total vitamin stored (milligrams per liver) followed the same pattern, and the correlation between liver vitamin A concentration on a fresh basis and total liver vitamin A was 0.96. The response of liver vitamin A concentration or total liver vitamin A to dietary retinyl palmitate levels was linear, and calculation of a curvilinear regression did not result in a significantly ($P < 0.05$) better fit of the data.

To estimate the conversion efficiency with which the carotenes present in corn silage were converted to vitamin A stored in the liver, the regression of total liver vitamin A stored on daily dietary retinyl palmitate intake was calculated, and the regression standard error of estimate was found to be 0.83. The regression equation fitted to the data by the method of least squares is illustrated in figure 1 and is as follows:

$$\hat{Y} = 1.6038 + 1.4516X$$

where $\hat{Y}$ = mg vitamin A/liver, and X = mg all-*trans*-retinyl palmitate ingested/lamb/day.

The correlation of total liver vitamin A stored with daily dietary retinyl palmitate intake was 0.74 and was statistically significant ($P < 0.01$).

The daily corn silage consumption provided 31.2 mg of total carotenes per lamb and resulted in liver storage of 12.3 mg of vitamin A. The relative biopotency of these carotenes as compared with dietary all-*trans*-retinyl palmitate was calculated as follows.

The liver vitamin A storage (12.3 mg) of sheep consuming 31.2 mg of corn silage carotenes per day was substituted for $\hat{Y}$ in the regression equation, and the daily dietary retinyl palmitate intake (X) which would have resulted in equivalent liver vitamin A storage was determined. The X value was found to be 7.4 mg. Assuming that, under these conditions, 31.2 mg of corn silage carotenes were equivalent to 7.4 mg of all-*trans*-retinyl palmitate, 1 mg of corn silage carotenes would be equivalent to 0.24 mg of all-*trans*-retinyl palmitate (7.4/31.2). Under the conditions used to establish the International Unit of vitamin A activity, 1 mg of all-*trans*-β-carotene was equivalent to 0.92 mg of all-*trans*-retinyl palmitate for the rat (12). Lambs used corn silage carotenes with 26% of this efficiency (0.24/0.92 × 100).

By converting 0.24 mg of all-*trans*-retinyl palmitate to International Units, 1 mg of corn silage carotenes was found to be equivalent to 436 IU. This relationship might be compared with the current recommendation of the NRC Subcommittee on Sheep Nutrition (13) that "a factor of 1 mg of β-carotene equal to 400–550 IU

TABLE 4

Liver vitamin A stores of lambs fed rations containing carotenes or retinyl palmitate for 70 days

	Lot 1	Lot 2	Lot 3	Lot 4	SE
	Corn silage + supplement	Basal + 1.1 mg retinyl palmitate/day	Basal + 3.3 mg retinyl palmitate/day	Basal + 9.9 mg retinyl palmitate/day	
Fresh liver, μg/g [1]	19.9	3.1 [2]	12.6 [3]	24.6	3.10
Dry liver, μg/g [1]	66.8	9.8 [2]	42.3 [3]	79.1	4.57
Total liver, mg/liver [1]	12.29	1.92 [2]	8.35 [3]	15.36	0.95

[1] Fourteen lambs per lot.
[2] Significantly lower ($P < 0.01$) than lots 1, 3 and 4.
[3] Significantly lower ($P < 0.01$) than lots 1 and 4.

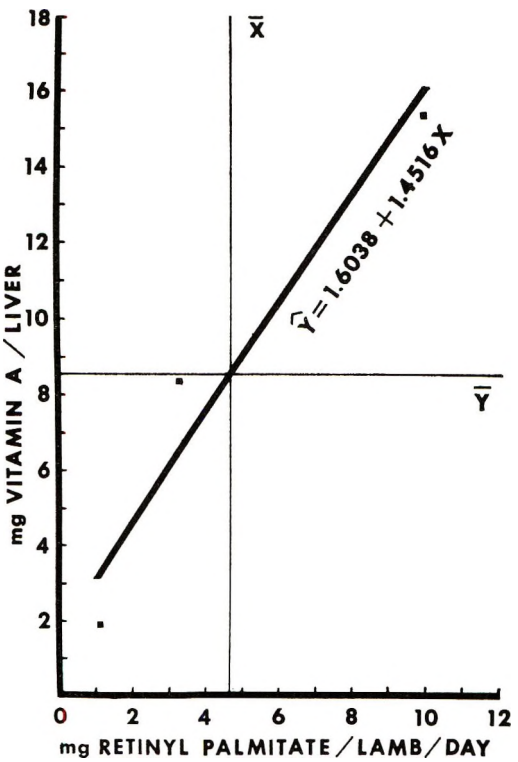

Fig. 1 Linear regression of total liver vitamin A stored (milligrams per liver) on daily dietary intake of retinyl palmitate per lamb.

vitamin A be used until more information is available."

It must be recognized that the relationship established between corn silage carotenes and dietary all-*trans*-retinyl palmitate may be appropriate only for the conditions of this study. It is not likely that carotene intakes which were either greater or lesser than those described would result in a linear dose-response curve with the same slope as that produced by retinyl palmitate. Lesser carotene intakes might conceivably result in greater conversion efficiency, whereas greater carotene intakes might be converted to vitamin A less efficiently. In this case, where corn silage containing 8.1 mg of total carotenes per kilogram was fed to the limit of appetite of 40-kg lambs, it appears that the more conservative NRC carotene conversion factor should be used.

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Preservation of Exocrine Pancreatic Function in the Magnesium-deficient Rat [1]

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ABSTRACT Pancreatic exocrine function was studied in young and adult rats which had been fed a magnesium-deficient diet, and compared with that of pair-fed, magnesium-supplemented controls, and with young rats fed a commercial laboratory ration on an ad libitum basis. No differences were demonstrated in respect to volume, amylase or nitrogen output between the secretions of the test or control animals, either in the resting state or after maximal stimulation with secretin and pancreozymin. Severe magnesium deficiency in young rats produced no depression in the magnesium content of the pancreas, although depletion of serum, red cells, bone, heart and skeletal muscle magnesium was achieved. Chronic dietary deficiency of magnesium in adult rats was not associated with depletion of pancreatic magnesium. The young animals showed the neuromuscular and cardiovascular complications of magnesium deficiency at a time when there was no decrease in pancreatic magnesium. Magnesium deficiency alone is unlikely to play a role in the depression of pancreatic function seen in the hypomagnesemic states which may accompany malnutrition.

In recent years an appreciable amount of data has become available concerning the essential role of magnesium in vitro as a cofactor in ATP-transferring enzymes, in protein synthesis and in oxidative phosphorylation (1). The clinical syndromes of magnesium deprivation in growing or adult animals and in man have been extensively reported and recently reviewed (2), and the effects of magnesium deficiency on the cation content of various tissues have been measured (3). Studies of various aspects of organ function in magnesium deficiency have been made in the thyroid (4), the heart (5, 6), skeletal muscle (7), the kidneys (8), the liver (9) and the gut (10, 11), but no studies have been made of the exocrine function of the pancreas in magnesium deficiency. Because this organ synthesizes more protein per gram of gland than any other body tissue (12), and requires metabolic energy to secrete water and bicarbonate (13), some abnormalities of pancreatic exocrine function might be predicted if the organ became sufficiently magnesium depleted. Menaker and Kleiner (14) demonstrated that protein-depleted rats failed to regain their weight if deprived of magnesium during the period of protein repletion, suggesting an impaired utilization or absorption of dietary protein in magnesium deficiency. Opie et al. (15) reported a patient with magnesium deficiency in whom impaired pancreatic function was demonstrated. If maldigestion does occur on the basis of inadequate pancreatic enzyme production due to magnesium deficiency, this is clearly of considerable importance, since such a lesion would enhance protein deficiency states, e.g., kwashiorkor, where magnesium deficiency is known to occur (16, 17).

In this study pancreatic function has been tested in young, actively growing rats made severely magnesium deficient, and in adult rats with chronic dietary deficiency of magnesium. The magnesium content of the pancreas has been measured, and the results are compared with magnesium-supplemented, pair-fed control animals, and with young animals receiving an ad libitum diet of commercial laboratory ration.[2]

METHODS

White, male rats of the Sprague-Dawley strain were used throughout; three groups of rats and their pair-fed controls were

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

studied. Group 1 comprised 6 young rats, initially weighing 100 to 110 g. They were caged individually and fed a commercially prepared, magnesium-deficient diet [3] which contains less than 1 mg of magnesium per 100 g diet by analysis in our laboratory. The diet contained: (in percent) casein (high protein), 25.00; choline chloride, 0.25; corn oil, 5.00; dextrose, 58.28; gelatin, 5.00; *l*-inositol, 0.10; DL-methionine, 0.30; and salt mix, 5.99. The salt mixture consisted of: (in grams) calcium carbonate, 905.84; calcium phosphate, 226.44; copper sulfate, 0.92; iron citrate, 84.52; manganous sulfate, 12.08; potassium iodide, 2.40; potassium dihydrogen phosphate, 981.16; sodium chloride, 507.20; and zinc carbonate, 0.76 per 45.5 kg of diet (18). Vitamins present were: (in milligrams per gram diet) biotin, 0.0003; vitamin B_{12}, 4.41×10^{-5}; calcium pantothenate, 0.025; choline chloride, 2.5; folic acid, 0.0025; inositol, 1.0; menadione, 0.005; nicotinic acid, 0.07; pyridoxine hydrochloride, 0.07; riboflavin, 0.006; thiamine hydrochloride, 0.006; α-tocopherol, 0.0202; and vitamins A and D, 40 and 8 units/g diet, respectively. The rats were allowed deionized, distilled water ad libitum, and studied after 21 days.

Group 2 comprised 5 adult rats, initially weighing 300 g. They were treated identically to the group 1 animals except that they were kept on the diet for considerably longer periods than the young rats, and killed after 90 to 120 days. Groups 3 and 4 were pair-fed controls for groups 1 and 2, respectively. They were matched for weight and sex and differed only from their pair-mates in receiving a daily injection of subcutaneous magnesium chloride (initially 40 mg, subsequently 70 mg), given as a 1.5% solution. Group 5 consisted of 5 young rats, weighing approximately 100 g, who had been fed a standard laboratory ration on an ad libitum basis; the laboratory ration contains 180 mg of magnesium per 100 g ration by analysis in our laboratory. All rats were weighed daily, and fasted for 12 hours before the pancreatic-function tests.

Pancreatic function was measured on a test rat and its control on the same day, using the same solution of freshly prepared secretin (Boots) and pancreozymin (Boots) for each pair member. The rats were anesthetized with 15% urethane intramuscularly, 1.3 mg/g body weight. Urethane produces some depression of pancreatic function (19); since test and control animals were treated identically, this limitation was considered acceptable. After the abdomen was opened and the common biliary-pancreatic duct identified, a fine polyethylene tube (Clay Adams, PE 10, O.D. 0.061 cm) was used to divert bile from the proximal common duct to the proximal duodenum, thus preserving its stimulating function and leaving the distal common duct to carry only pancreatic secretion. The most distal portion of the duct was cannulated with the polyethylene tubing to a distance of 2 mm, and the cannula tied firmly in place with a silk ligature. The abdomen was closed in two layers and the cannula connected to a graduated polyethylene tube. A polyethylene cannula was inserted into an exposed femoral vein as a route for the injections of pancreozymin and secretin, and kept patent with a saline infusion.

Pancreatic secretion was collected in the polyethylene tube for the initial 30-minute period and is called "resting" juice. A "maximal" stimulus of pancreozymin was given intravenously, in 2 ml of 0.15 M saline, over a 2-minute period. In the young rats, a dose-response curve indicated that a maximal response occurred with 0.25 Crick-Harper-Raper unit and for the adults with 0.50 unit. Ten minutes later, a maximal stimulus of secretin, 0.50 Crick-Harper-Raper unit for either young or adult rats, in 2 ml of 0.15 M saline, was given intravenously over a period of 2 minutes and the secretion was collected for a period of 80 minutes. The volume of juice collected in the total 90 minutes following the injection of pancreozymin was measured and called "stimulated" juice. After collection, all juice was frozen at − 20° and amylase content was estimated within 24 hours.

For both "resting" and "stimulated" juices, the total volume in microliters was measured using the graduated polyethylene tube. The total output of nitrogen in micrograms was measured by a micro-

[3] General Biochemicals Inc., Chagrin Falls, Ohio.

Kjeldahl method and nesslerization; and total output of amylase was assayed by an amyloclastic method as modified by King and Wootton (20). A unit of amylase is defined as the amount of enzyme which hydrolyzes 5 mg of starch in 15 minutes at 37°. The pancreatic juice was diluted $1:10^3$–10^5 in 0.15 M NaCl; amylase activities were directly proportional to dilution over a threefold range in the middle of the calibration curve. Repeatability of the method is 10%.

At the conclusion of the pancreatic test, the rats were exsanguinated by cardiac puncture and the blood collected in a heparinized syringe. The blood was separated immediately and the plasma frozen. The red cells were washed 3 times in cold 0.15 M saline and a 1:1 lysate made with distilled water. Plasma and red cell magnesium levels were determined by atomic-absorption spectroscopy, using the method of Iida et al. (21). The liver, heart, pancreas, duodenum, a portion of thigh muscle and the right femur were removed, the soft tissues weighed and homogenized in an all-glass, metal-free homogenizer, and 10% dilutions in metal-free distilled water were made. Aliquots of the solutions were then wet-ashed in a 2:1 mixture of nitric and perchloric acid, the resulting ash being dissolved in 0.1 N hydrochloric acid. The femur was cleaned of adherent muscle by immersion in hot distilled water for 2 minutes and then oven dried and ashed in a silica crucible for 16 hours at 500°; the ash was dissolved in hot 2 N hydrochloric acid. Magnesium contents of all tissues were determined by atomic-absorption spectroscopy using the method of Iida et al. (21). Statistical differences were assessed by the *t* test (22); a significant difference is defined as a *P* value of less than 0.05. The repeatability of the magnesium method is 2%; duplicate tissue analyses differed on the average by 5%. Accuracy was established on each set of analyses by measuring the magnesium content of a National Bureau of Standards sample of dolomite; the results varied from 95 to 105% of the given values.

RESULTS

In young rats, signs of magnesium deficiency, i.e., cutaneous hyperemia and encrusted hairless lesions on the skin and back, were seen within 10 days. The animals were listless, ate poorly and gained weight slowly. Within 20 days they became hyperexcitable, and developed convulsions. Table 1 shows the means and SD's of the test and control rats' weights and their plasma and red cell magnesium concentrations at the time of the pancreatic function test, after 21 days on the diet. Although pair-feeding was successful in limiting the weight gain of the controls to that of the test animals, the plasma and red cell magnesium values for the control animals on 40-mg supplements averaged 1.2 and 2.4 mEq/liter which is lower than the average values for young rats fed the laboratory ration (table 1). In the last two controls, the supplement was increased to 70 mg of magnesium/day, and these two animals had plasma magnesium val-

TABLE 1

Mean weight, plasma and red cell magnesium levels in young and adult rats, their magnesium-supplemented, pair-fed controls, and young rats fed ad libitum a commercial laboratory ration [1]

Diet	No.	Weight	Plasma magnesium	Red cell magnesium
		g	*mEq/liter*	*mEq/liter*
		Young rats		
Magnesium-deficient	6	114 ± 20	0.5 ± 0.1 [2]	1.8 ± 0.1 [2]
Magnesium-supplemented	6	120 ± 18	1.5 ± 0.5	3.0 ± 1.0
Laboratory ration	3	110 ± 13	1.9 ± 0.12	4.1 ± 0.17
		Adult rats		
Magnesium-deficient	5	400 ± 40	1.3 ± 0.2	3.8 ± 0.1 [2]
Magnesium-supplemented	5	406 ± 38	2.3 ± 0.26	5.3 ± 0.3

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[2] $P < 0.01$ compared with magnesium-supplemented, pair-fed controls in young rats.

ues of 1.9 mEq/liter and 2.0 mEq/liter, and red cell magnesium of 4.0 mEq/liter. Neither the 40- nor the 70-mg magnesium-supplemented animals showed any sign of magnesium deficiency.

The adult rats did not manifest the gross signs of magnesium deficiency seen in the young. After 3 to 4 weeks there was a mild auricular hyperemia, which tended to fade. Weight gain was slow but persistent, averaging 100 g and the animals were killed after an average of 105 days on the diet. Table 1 shows their weights and the plasma- and RBC-magnesium levels at the time of the pancreatic-function test. A moderate hypomagnesemia of both the plasma and red cells has been achieved in the test group.

The results of pancreatic function are shown in table 2. In the young rats a two-fold increase over the resting level of volume and amylase output occurs with maximal secretin and pancreozymin stimulation of the control animals. There is no significant difference, however, between the magnesium-deficient and the control animals in respect to any measured parameter of pancreatic function. The nitrogen output was increased in the test animals but the differences were not significant. The two rats supplemented with 70 mg of magnesium showed no differences from those supplemented with 40 mg of magnesium.

For the adult rats the volume, amylase and nitrogen output of the test animals is actually more than that of the controls; these differences are not statistically significant with the exception of nitrogen output, which is higher for the test animals ($P < 0.01$). No instance of impairment of pancreatic function occurred in any of the hypomagnesemic rats.

Table 3 shows the magnesium contents of the various tissues of the test and control animals. For the young rats there is a 15% reduction in the magnesium content of skeletal muscle, a 14% decrease in heart, and a 12% decrease in the livers of the test animals compared with controls, but neither the pancreas nor duodenum shows any significant differences. The femur shows marked magnesium depletion.

In the adults, despite the mild hypomagnesemia, no significant reduction in the magnesium content of any tested organ was found. Because magnesium deficiency can alter the potassium, sodium and calcium contents of various tissues, these were measured in three of the five pairs but no significant changes occurred in the pancreas or other organs.

DISCUSSION AND CONCLUSIONS

Growing rats, facing a magnesium deficiency so severe that the deprived animals were convulsing (and dying) within 2 weeks of commencing the diet, had no demonstrable depression of pancreatic function. Small diminutions in magnesium contents of heart, skeletal muscle and

TABLE 2

Pancreatic function in young and adult rats, their magnesium-supplemented, pair-fed controls, and young rats fed ad libitum a commercial laboratory ration [1]

	No.	Volume		Amylase output		Nitrogen output
		Resting, 30 min	Stimulated, 90 min	Resting, 30 min	Stimulated, 90 min	Stimulated, 90 min
		µl		*Somogyi units*		*mg*
		Young rats				
Magnesium-deficient	6	15 ± 5	86 ± 14	1200 ± 440	8750 ± 4810	519 ± 250
Magnesium-supplemented	6	14 ± 3	78 ± 18	1260 ± 750	7500 ± 3410	380 ± 80
Laboratory ration	5	13 ± 4	80 ± 23	1390 ± 365	8176 ± 4220	432 ± 321
		Adult rats				
Magnesium-deficient	5	18 ± 5	165 ± 34	560 ± 190	7280 ± 3000	1353 ± 555 [2]
Magnesium-supplemented	5	19 ± 6	123 ± 22	456 ± 200	5200 ± 1600	801 ± 175

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[2] $P < 0.01$.

TABLE 3

Magnesium content of various organs from young and adult rats, their magnesium-supplemented, pair-fed controls, and young rats fed ad libitum a commercial laboratory ration [1]

Diet	No.	Muscle	Heart	Liver	Duodenum	Pancreas	Femur [2]
		mg/g N	*mg/g N*	*mg/g N*	*mg/g N*	*mg/g N*	*mg/g N*
				Young rats			
Magnesium-deficient	6	5.5 ± 0.2 [3]	6.1 ± 0.5	5.2 ± 0.8	7.4 ± 1.8	9.2 ± 1.2	0.252 ± 0.060 [4]
Magnesium-supplemented	6	6.5 ± 0.5	7.1 ± 0.9	5.9 ± 0.9	7.1 ± 1.1	9.4 ± 2.1	0.403 ± 0.060
Laboratory ration	3	6.5 ± 0.6	6.6 ± 0.4	6.0 ± 0.5	6.8 ± 0.3	8.2 ± 0.6	0.453 ± 0.320
				Adult rats			
Magnesium-deficient	5	7.8 ± 0.6	6.9 ± 0.4	7.0 ± 0.9	8.7 ± 1.7	9.3 ± 0.8	0.687 ± 0.453
Magnesium-supplemented	5	8.0 ± 0.2	7.5 ± 0.4	7.0 ± 0.6	8.0 ± 1.4	9.7 ± 0.8	0.754 ± 0.762

[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[2] Percent of ash weight.
[3] $P < 0.05$.
[4] $P < 0.01$ compared with magnesium-supplemented, pair-fed controls in young rats.

liver were found but even in the presence of very serious reductions in plasma and red cell magnesium, the magnesium content of the pancreas remained unchanged, presumably explaining the preservation of pancreatic function. In addition, the function of the isolated rabbit pancreas is not impaired by incubation in a Krebs-Ringer solution free of magnesium,[4] although a sodium or bicarbonate-free incubation solution markedly impairs secretion (13).

As found by others (3), clinical symptoms of magnesium deficiency develop slowly in the 200-g rat, although it is possible to achieve some degree of chronic hypomagnesemia. Depletion of magnesium was not found in any of the tissues studied in 300-g rats and, as in the acutely depleted rats, no impairments were found in pancreatic function.

We are unable to find previous information concerning the magnesium content of rat pancreas, either normal or magnesium depleted, but in common with Kessner et al. (10) and Lifshitz et al. (11), we were not able to demonstrate a reduction in magnesium content of small bowel from the depleted animals. This "avidity" for body magnesium stores thus appears to be true of the pancreas also.

Three general conclusions can be drawn from these studies. First, in the magnesium-depleted rat, failure to gain weight is not on the basis of a magnesium deficiency-induced pancreatic malfunction; this organ is shown to respond normally at a time when the animal is very ill from the magnesium deficiency and likely to die from complications. The failure of weight gain may be on the basis of inadequate food intake, associated with the hyperexcitability and tetany of the animals.

Second, it has been demonstrated that protein depletion per se can impair pancreatic structure and function (23–25). This alone could account for the clinical observation of pancreatic insufficiency in kwashiorkor and like states, irrespective of the magnesium balance. Finally, it seems likely that before levels of tissue magnesium could be reduced to such concentrations that the amounts necessary as cofactors or catalysts in protein synthesis become inadequate, the animal would already have died of the neuromuscular or cardiovascular complications. Thus, while magnesium deficiency is important in malnourished states, it is unlikely to be directly responsible for the pancreatic dysfunction occasionally found in these conditions.

It is still possible that magnesium deficiency potentiates the effects on the pancreas of protein (14) and potassium deficiencies, which also occur in states of malabsorption or malnutrition. This possibility remains to be tested in future experiments.

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ERRATUM

Radhakrishnamurty, R., J. F. Angel and Z. I. Sabry 1968 Response of lipogenesis to repletion in the pyridoxine-deficient rat. J. Nutr., 95: 341. In the first column of table 2 "Liver wt, g/100 g body wt" and "Epididymal pad wt, g/100 g body wt" are reversed in two places.

To correct these errors in your copy of volume 95, number 3, please cut along lines of reprinted section below and paste over entries 4 and 5, 9 and 10, table 2, page 344.

Liver wt, g/100 g body wt	4.11 ± 0.21	3.46 ± 0.2	5.58 ± 0.13 [5]	3.72 ± 0.12	3.20 ± 0.07 [5]	3.76 ± 0.24
Epididymal pad wt, g/100 g body wt	0.60 ± 0.08	0.58 ± 0.06	0.84 ± 0.10	0.81 ± 0.05	0.69 ± 0.03	1.04 ± 0.08
Liver wt, g/100 g body wt	4.49 ± 0.11	3.87 ± 0.23	6.13 ± 0.28 [5]	3.95 ± 0.16	3.48 ± 0.11	4.65 ± 0.14 [5]
Epididymal pad wt, g/100 g body wt	0.62 ± 0.05	0.65 ± 0.05	0.86 ± 0.04 [5]	0.73 ± 0.07	0.88 ± 0.04	0.93 ± 0.11