Autoradiographic Patterns of Methionine-2-^{14}C and Methionine-Methyl-^{14}C in Tissues of the Adult Rat [1,2]

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ABSTRACT Using Kodak Type A autoradiographic plates, autoradiograms were prepared of 20 to 25 tissues from adult male Wistar rats killed 4 and 24 hours after feeding methionine-2-^{14}C and 24 hours after feeding methionine-methyl-^{14}C. The autoradiographic method used permitted the viewing on the same slide of the tissue alone, the autoradiogram alone and the tissue superimposed on its autoradiogram. With the exception of bone, all tissues produced autoradiograms at both time intervals; however, the 4-hour autoradiograms were much less intense than those produced after 24 hours. Epithelial tissue and bone marrow produced very intense autoradiograms. Muscle and connective tissue exhibited little activity. The uniformity of labeling observed when methionine-2-^{14}C and methionine-methyl-^{14}C were fed is interpreted to reflect the broad distribution of methionine and its metabolites in tissue proteins and other macromolecules rather than the existence of selective priority sites for compounds formed from its methyl or α-carbon moieties.

The quantitative requirements for amino acids for protein synthesis and the amino acid composition of tissues have been well established. However, few reports have appeared in the literature concerning the relative localization of an amino acid in different parts of a tissue. In the case of methionine, this is of particular interest, as its sulfur, 4-carbon chain, and labile methyl group follow other metabolic pathways in addition to general protein synthesis.

It seemed worthwhile, therefore, to determine whether the amino acid was uniformly distributed in certain tissues, or whether specific parts of organs assumed priority in the uptake of the amino acid.

The development of an autoradiographic method for microsections of tissues permitted viewing the stained tissue section and the autoradiogram of the tissue section separately and while superimposed on each other. This technique was used in the present study to investigate possible differences in the autoradiographic patterns of tissues of adult rats following administration of methionine-2-^{14}C or methionine-methyl-^{14}C.

Autoradiographic techniques have been used after the injection of methionine-^{35}S to study sulfur deposition in tissues (1–3), sites of protein synthesis (4, 5), methyl transfer to ribonucleic acid (6), and protein metabolism (7, 8).

EXPERIMENTAL PROCEDURE

Following a 10-hour fast, adult, male rats of the Wistar strain were fed by stomach tube 18 mg of either DL-methionine-2-^{14}C or L-methionine-methyl-^{14}C with specific activities of 0.57 and 1.84 mc/mmole, respectively. The labeled methionine was fed as a component of a semipurified, homogenized diet which provided all of the essential nutrients required by the rat (9). Of 2 rats fed methionine-2-^{14}C, one was killed 4 hours after the administration of the diets; the other at 24 hours. The rat that received methionine-methyl-^{14}C was killed 24 hours after feeding.

At the time of this investigation L-methionine-2-^{14}C could not be obtained commercially nor was it feasible to synthesize this compound in our laboratory. Differences in transport of the D-isomer of methionine would affect interpretation of localization patterns at early time intervals

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but would not be of significance after 4 or 24 hours.

After the administration of the diet, water was provided ad libitum. At the time of killing, the animals were anesthetized with ether and exsanquinated by cardiac puncture. Tissues were removed quickly and fixed in Bouin's solution. Following routine dehydration and paraffin embedding, autoradiograms of tissue sections were made on Kodak Type A autoradiographic plates, using the method of Evans and McGinn (10).

Autoradiograms were made of 20 to 25 tissues from each rat. Tissue sections were cut at a thickness of 8 μ. After a sufficient exposure time, which ranged from 6 to 22 weeks in a light-tight box, the preparations were developed and stained with hematoxylin and eosin. Tissues from a control rat fed a stock ration were exposed in the same manner.

RESULTS AND DISCUSSION

Gastrointestinal tract. Throughout the alimentary tract there was a heavy deposition of radiocarbon in the epithelial linings from the esophagus through the large intestine. The mucosa exhibited more activity than other coats (figs. 1–3, 10 and 11); there was moderate activity in the submucosa and very little in the muscularis. Measurement of the radiocarbon content of proteins isolated from the various segments of the alimentary tract showed the highest incorporation of methionine to have occurred in duodenum and jejunum, both initially and after 4 and 24 hours.

In liver, at 4 hours, the autoradiogram was more intense in some areas around the larger vessels in the hepatic portal area, in others around the central vein. At 24 hours, radiocarbon was uniformly distributed in hepatic lobules (fig. 12). We have demonstrated previously that significantly smaller concentrations of the α-carbon of methionine than its methyl carbon occur in liver 16 to 30 minutes following the administration of an aqueous solution of methionine and 24 hours after the feeding of methionine as a component of a semi-purified diet (11). The heavier deposition of radiocarbon around hepatic vessels at 4 hours may represent methionine or a compound containing its α-carbon prior to entry into blood vessels for transport to cells. Zbarskii and Perevoshchikova (8) and Leblond and co-workers (4) have observed that the rate of inclusion of labeled methionine into proteins of cell nuclei of normal liver is below that occurring in proteins of the whole tissue.

Activity was generalized in the cells of pancreatic acini. No difference was detected in the uptake of radiocarbon in the islets versus other pancreatic tissue. Measurements of radiocarbon at various time intervals in the whole tissue showed a very high accumulation of ^{14}C at 30 minutes which fell to low levels at 4 and 24 hours. As these data paralleled activities in pancreatic proteins isolated from the tissue at these same intervals,[3] it was concluded that methionine was used for the synthesis of pancreatic secretions of a protein nature. Leblond and co-workers (4) have demonstrated that the synthesis of these pancreatic secretions is localized in the zymogen granules of acinar cells, which serve as precursors of pancreatic enzymes. Four hours following the injection of methionine-^{35}S these investigators observed an intense autoradiographic response of the material in the lumen of excretory ducts of pancreatic tissue of rats.

In the submaxillary gland, activity was also uniformly distributed. More radiocarbon was present in the serous secretory units than in mucous secretory units. After incubation of salivary gland from *Smittia* sp. (Diptera) with methionine-methyl-^{14}C, Sirlin and co-workers (6) observed about the same amount of acid-insoluble label per unit area in the nucleolus, chromosomes and cytoplasm. Radioactivity was not due on the whole to methionine bound to RNA but was in RNA itself. Their results suggested a direct transfer of methyl-^{14}C from methionine to nucleolar RNA.

Reproductive system. At 24 hours, in the testis activity was observed in the seminiferous tubules, in interstitial tissue, and in extravasated fluid in the periphery of the organ. Testicular tissue at 4 hours produced a very faint autoradiogram; the tissue between tubules, however, was not active. The autoradiograms produced by

[3] Edwards et al., unpublished data.

spermatogonia and primary spermatocytes appeared to be more intense than those of other spermatogenic cells (fig. 13).

At 4 hours, in the prostate, there was activity in the alveolar cells and none in the secretion. Both alveolar cells and secretion contained radiocarbon at 24 hours. When methionine-2-^{14}C was administered, where the secretion stained pink, an intense autoradiogram was produced. In those alveoli where the secretion stained blue, a lighter autoradiogram occurred (fig. 14). In the prostatic tissue of the rat fed methionine-methyl-^{14}C, there was no variation in the staining of the secretion, and radiocarbon appeared to be uniformly deposited in the alveoli.

Respiratory and excretory systems. The epithelial lining of the trachea was very radioactive at 4 and 24 hours after administration of methionine-2-^{14}C (figs. 1, 2 and 3). Activity was uniformly distributed in lung alveoli.

In kidney, more activity was observed in the renal cortex than in the medulla in all rats. After 4 hours, the autoradiogram was light. The bulk of the activity was observed in the uriniferous tubules, with glomeruli appearing as pale areas in some instances (figs. 4–6). The appearance of greater activity in these tubules may represent the presence of radiocarbon prior to its excretion in urine, rather than actual tissue uptake of labeled methionine.

Activity in the urinary bladder was concentrated in the epithelial lining and desquamated epithelium in the lumen.

Endocrine system. The autoradiogram was intense, and activity was uniformly distributed in all zones of the adrenal cortex. The medulla had little activity (fig. 7). The cortex is the site of synthesis of 26 or more adrenal hormones. It is well known that epinephrine is produced in the medulla, and that it receives a methyl group from methionine. The intense autoradiogram produced over the adrenal cortex may indicate the utilization of methionine for synthesis of other adrenal hormones, as well as its utilization for general tissue synthesis.

Autoradiograms of pituitary showed dense uptake of methionine in the anterior lobe, moderate distribution in the intermediate lobe, and light deposition of the radiolabel in the posterior lobe (fig. 17). In both adrenal and pituitary, the difference in autoradiographic patterns within the tissue may reflect different rates of turnover of protein components.

In the thyroid, radiocarbon was localized in the cells of the follicles in all rats. The colloid was devoid of activity (figs. 1, 2 and 3). In contrast, Leblond and co-workers (4) have observed intense autoradiograms over the colloid of most thyroid follicles when ^{35}S-methionine was injected. Merkulov (2) has reported that ^{35}S-methionine is localized in thyroid tissue via the process of protein secretion into the follicular volume followed by resorption.

At both 4 and 24 hours, the distribution of activity in thymus was uniform.

Circulatory system. Radiocarbon was evenly deposited in heart muscle and connective tissue. Activity in this organ was light. Blood cells and plasma in several of the organs appeared radioactive for all animals. Blood vessels were radioactive in many organs. A heavy concentration of radiocarbon was observed in spleen. The red pulp appeared to be more active than the splenic nodules. Bone marrow was very radioactive; however, bone tissue was free of activity (fig. 15). Similar results for bone and bone marrow have been reported by Leblond and co-workers (4).

The intense autoradiogram produced by bone marrow was correlated with other data which demonstrated the occurrence of high concentrations of radiocarbon at 30 minutes, 4 and 24 hours (9, 11–13) in this tissue. The rat appears to incorporate into bone marrow tissue, and its isolated protein, compounds originating from DL-methionine-methyl-^{14}C equally as well as those from its L-isomer. One pathway for the utilization of the methyl group of methionine is for the synthesis of thymine, a component of deoxyribonucleic acid (DNA). Cells rich in nuclear material, such as those in bone marrow, have larger concentrations of DNA.

Other tissues. Very little activity was observed in muscle. The radiocarbon present was distributed uniformly (figs. 1–3 and 10). As indicated previously, no ac-

tivity could be detected in bone or cartilage (figs. 1–3 and 15).

In skin (figs. 7–9), at 24 hours radiocarbon was concentrated in hair and hair follicles, producing an intense autoradiogram. Some activity was observed in the epithelium and blood vessels of skin. After 4 hours, there was very slight indication of activity in these structures. Leblond (4) has observed intensely radioactive hair follicles following the injection of methionine-^{35}S in rats.

It has been demonstrated by du Vigneaud and his co-workers (14) that the sulfur of methionine, but not its carbon chain, is utilized in the synthesis of cystine. These autoradiograms, therefore, demonstrate only the presence of methionine in hair and hair follicles, rather than methionine and cystine.

In comparison with other tissues, the deposition of radioactivity in brain was light. Activity was heaviest in the granular layer of cerebellum, moderate in the molecular layer, and light in the white matter (fig. 18).

No autoradiograms were produced by any of the non-radioactive tissues exposed to autoradiographic plates.

Epithelial linings and glandular tissues such as liver, adrenal, pituitary, and pancreas, which are composed mainly of epithelial cells, exhibited much more activity than did other tissues. Epithelium has a marked capacity for regeneration; the rapid active mitotic proliferations could be a factor to explain the high uptake of the amino acid. Leblond et al. (4) and Bélanger (5) have reported similar observations and attribute the intense staining of epithelium and bone marrow to the high rate of cell production by these tissues.

The existence of radiocarbon in all tissues, except bone, indicates that protein synthesis is widespread in contrast with being limited to a few specific tissues. Leblond and his colleagues (4) arrived at similar conclusions. As they observed no activity or slight activity over nuclei, these workers concluded that the cytoplasm of all observed cells rather than nuclei may synthesize protein.

The absence of significant concentrations of radiocarbon in bone may be due to the low turn-over rate of this tissue. The patterns of deposition of radiocarbon were similar when methionine-2-^{14}C or methionine-methyl-^{14}C supplied the source of radiocarbon. Also, the positions of localization of activity in tissues 4 hours after administration of the radiomethionine were the same as for tissues taken after 24 hours; however, the latter tissues contained much more radiocarbon.

It is well known that the methyl group of methionine may undergo a large number of metabolic transformations, both via transmethylation and 1-carbon metabolism. Similarly, the carbon skeleton of the amino acid is subject to a variety of reactions, although the products are less well defined. The compounds resulting from these transformations may then be incorporated into many types of macromolecules. It appears likely, however, that the uniformity of labeling observed in most tissues reflects, primarily, the incorporation of methionine and amino acids formed from its methyl and α-carbons into protein. In addition to the labeling of protein, the various metabolic products containing the radiolabel may be distributed randomly in tissues.

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PLATE 1

EXPLANATION OF FIGURES

The figures in plate 1 are shown in sets of three. The tissue alone is shown in the first column; in the second column, the tissue is superimposed on its autoradiogram; in the third column appears the autoradiogram only of the tissue shown in the first column. × 16. The tissues are from a rat killed 24 hours after feeding DL-methionine-2-^{14}C.

1,2,3 Cross section through thyroid region showing thyroid (thy), trachea (tra) and esophagus (eso). Note the heavy activity in the epithelial lining of trachea and esophagus and in mucous glands outside trachea. There is moderate activity in the thyroid and less in muscle. Note the complete absence of activity in cartilage.

4,5,6 Kidney. Radioactivity is heavy in the cortex, moderate in the medulla.

7,8,9 Skin. Radioactivity is concentrated in hair follicles. There is moderate activity in the surface epithelium and blood vessels.

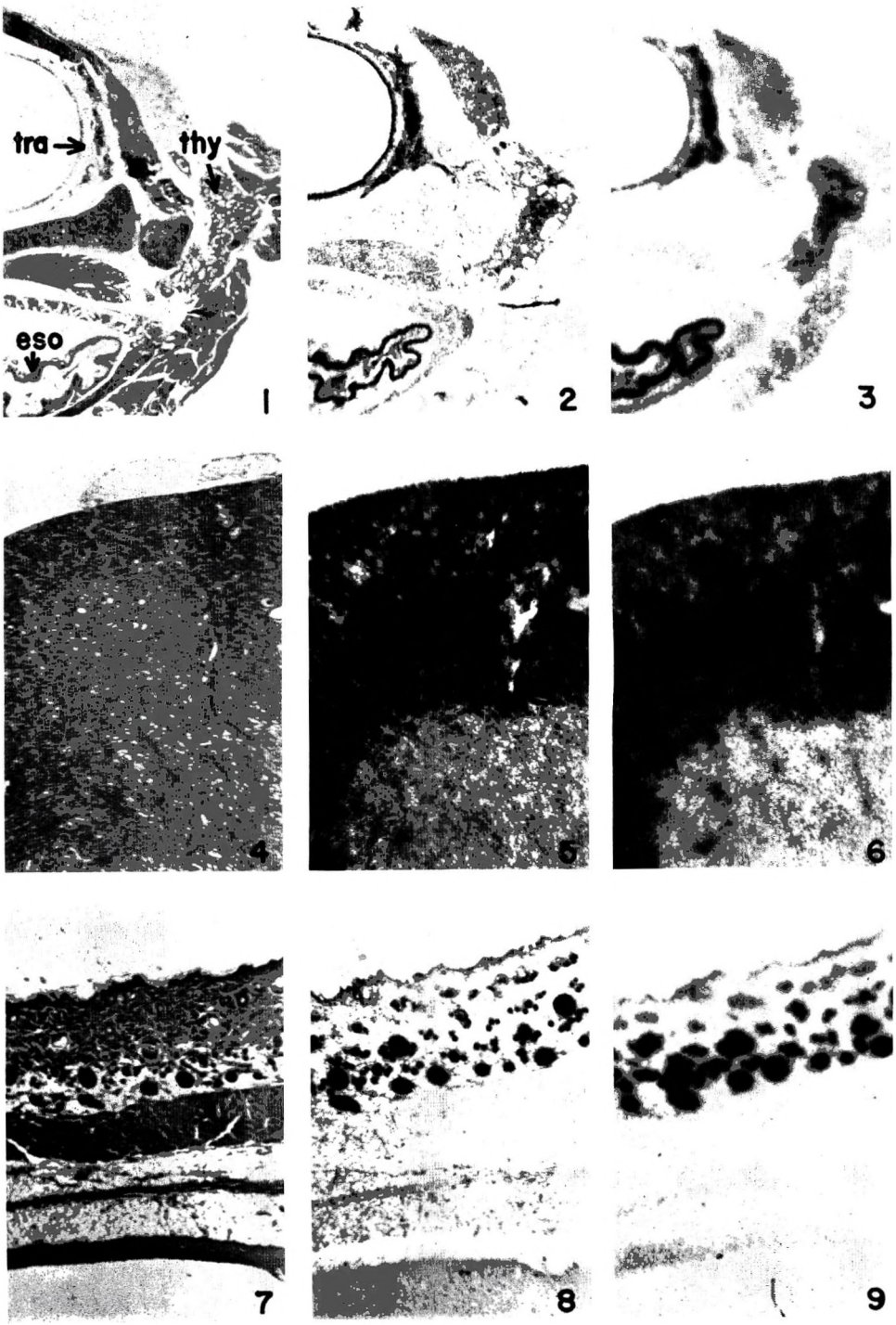
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PLATE 2

EXPLANATION OF FIGURES

All figures shown in plate 2 are photographs of the tissue section superimposed on its autoradiogram. × 16. The tissues were taken 24 hours following feeding of methionine-2-^{14}C (11–14, 18) and methionine-methyl-^{14}C (10, 15–17).

10 Stomach. There is heavy activity in the mucosa, light activity in the submucosa and muscularis.

11 Large intestine. There is heavy activity in the mucosa, light activity in the submucosa and muscularis. Note that the fecal material in lumen is also radioactive.

12 Liver. There is a heavy deposition of radiocarbon uniformly distributed throughout liver.

13 Testis. There is moderate activity in the tubules. Note the heavy activity in the fluid near the periphery of the organ.

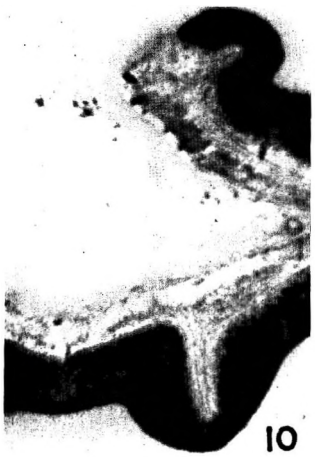
14 Prostate. There is a heavy deposition of radiocarbon in the secretion found in some of the alveoli whereas there is only a moderate amount in others.

15 Bone (longitudinal section). Bone marrow is very radioactive, whereas bone is devoid of activity. There is light-to-moderate activity in the muscle adhering to bone.

16 Adrenal. There is heavy activity in the cortex, moderate activity in the medulla.

17 Pituitary. There is heavy activity in the anterior lobe, moderate activity in the intermediate lobe and light activity in the posterior lobe.

18 Brain. Activity is heaviest in the granular layer of the cerebellum, moderate in the molecular layer and light in the white matter.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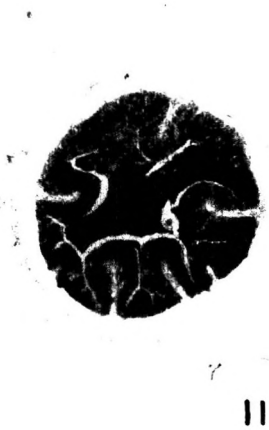
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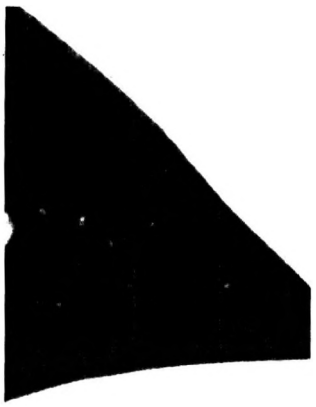
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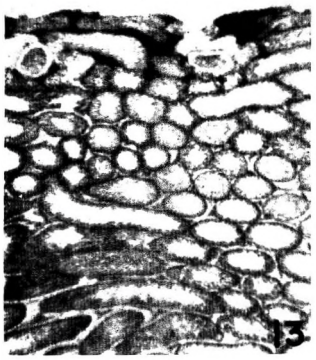
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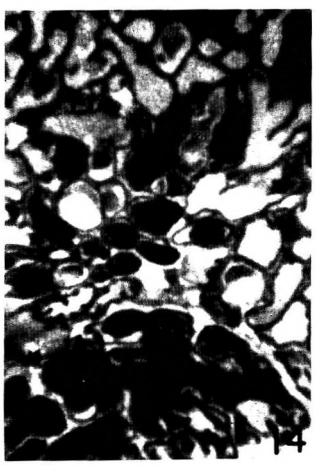
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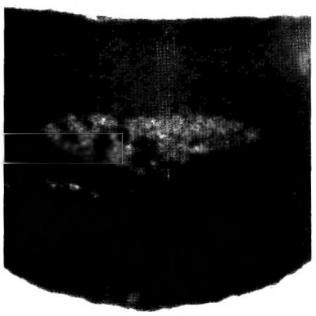
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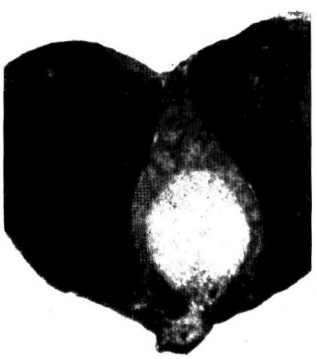
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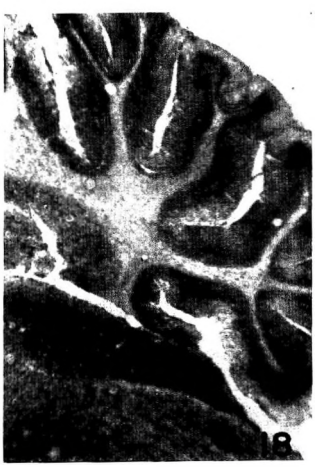
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Comparison of the Hypercalcemic Action of Vitamins D_2 and D_3 in Chicks and the Effect on Tetracycline Fixation by Bone [1]

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ABSTRACT Doses of crystalline vitamins D_2 and D_3 ranging from 3 IU/day vitamin D_2 and 40 IU/day vitamin D_3 to 40,000 IU/day of each vitamin were administered daily to chicks fed a rachitogenic diet and the effects were noted on body weights, serum calcium and phosphorus, tetracycline uptake and bone microradiographic appearance. Although body weight and bone weight data indicated that vitamin D_3 was approximately 10 times more toxic than vitamin D_2 (about the same as the antirachitic potency difference), relative toxicity ratios could not be estimated from the effects on serum calcium level, or on bone ash. At all dose levels except at 40,000 IU/day, the vitamin D_3 gave a higher percentage of bone ash than vitamin D_2. No tetracycline was taken up by bones of chicks given 40,000 IU/day of vitamin D_3 indicating no new bone deposition and a high degree of resorbing activity in these bones.

In a recent study (1) of the effects of crystalline vitamins D_2 and D_3 on the bone ash, and serum calcium and phosphorus levels in rachitic chicks, it was observed that the antirachitic potency of vitamin D_3 was 8 to 11 times greater than that of vitamin D_2 in effecting partial (50%) healing. This observed efficacy ratio was smaller than most previously reported ratios. A qualitative difference also was noted, in that this potency or efficacy ratio apparently increased as an attempt was made to effect more complete healing of rickets.

Since the D vitamins produce toxic effects, including hypercalcemia, when administered in large doses, the investigation of their relative potencies in chicks has been extended to include the "toxic" dose range in order to evaluate whether the potency difference between vitamins D_2 and D_3 continues to hold. As was true for anti-rachitic doses of vitamin D, few data are available concerning serum calcium and phosphorus changes in a systematic dosing schedule utilizing the crystalline vitamins.

In the present study, doses of crystalline vitamins D_2 and D_3 ranging from 3 IU/day vitamin D_2 and 40 IU/day vitamin D_3 up to 40,000 IU/day of either vitamin were administered daily to chicks fed a rachitogenic diet; the effects were noted on body weights, fat-extracted bone weight, serum calcium and phosphorus levels, percentage of bone ash, tetracycline uptake and the microradiographic appearance of long bone shafts.

METHODS

The experimental schedule for producing rachitic chicks has been described previously (1). Male, White Leghorn chicks (Mt. Hope Queens) were obtained at one day of age and separated into groups of 8 to 14 animals. They were kept in a room from which all ultraviolet irradiation was excluded by red cellophane and were fed a commercial "rachitogenic chick test diet" (1.43% calcium, 1.10% phosphorus).[2]

After the chicks had been fed this rachitogenic diet for 21 days, each chick was intubated daily with 0.1-ml doses of sesame oil with either nothing added (rachitic control), or containing graded doses of vitamin D_2 (3, 10, 40, 100, 1000, 10,000 and 40,000 IU/day) or vitamin D_3 (as above but starting with the 40 IU/day level).[3] Treatment was continued for 14 days and the birds were weighed at inter-

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[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York.

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

[3] Crystalline vitamins D_2 and D_3 purchased from Mann Research Laboratories, Inc., New York.

vals. Three birds from each group were then injected intramuscularly with 10 mg tetracyline [4] 38 to 42 hours prior to killing. Food was withdrawn 24 hours prior to killing.

The chicks were decapitated and blood was withdrawn from the severed neck. Serum calcium and serum phosphorus levels were analyzed by methods referred to previously (1). The 2 tibia from each chick were dissected out; one was analyzed for percentage of bone ash, the other was fixed in absolute ethanol for microradiography and examination of fluorescence. The ethanol was replaced by methyl methacrylate monomer for 24 hours and then each bone was imbedded in polymerized methyl methacrylate. Sections (approximately 100 μ) were cut from each tibia by a diamond saw. These sections were photographed under ultraviolet light to determine the localization of tetracycline fluorescence, using a Zeiss microscope fitted with 35 mm camera and 6.5 × objective

[4] Liquamycin (oxytetracycline·HCl. 50 mg/cm³), Charles Pfizer and Co., New York.

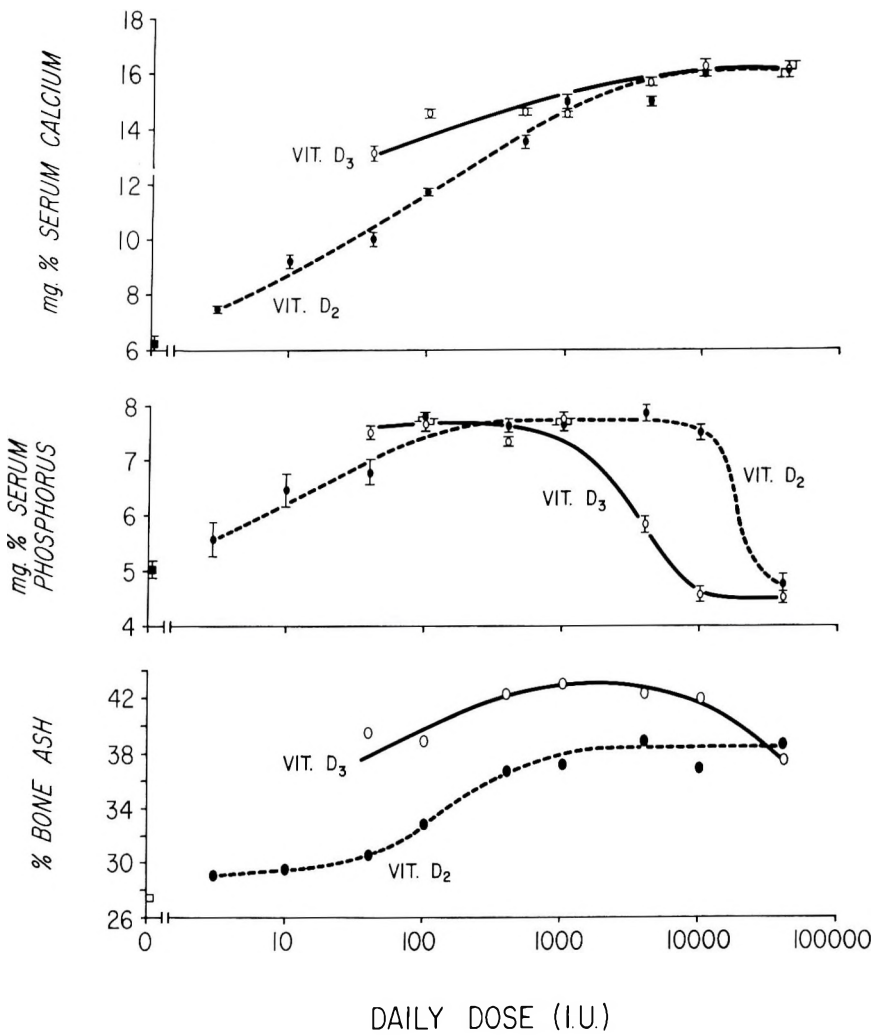

Fig. 1 Effect of vitamins D_2 and D_3 on: (top) serum calcium level; (middle) serum phosphorus level; and (bottom) % bone ash. Calcium and phosphorus values given as means ± SE.

(primary filter UG 1, secondary filter no. 50, film: Kodak high speed Ektachrome, A.S.A. 160). Six-minute exposures were used.

Microradiographs were prepared according to the usual procedures. Kodak type 649-0 Spectroscopic Plates (1″ × 3″ × 0.040″) were exposed for 10 minutes using these settings: filament, 2.55 amp; high voltage, 1200; plate current, 20 ma. The plates were developed for 5 minutes in Kodak D-19, and fixed for an equal period of time.

RESULTS

Although firmly established and accepted as a reliable indicator of antirachitic activity of vitamin D, the percentage bone ash does not appear to be a reliable indicator of the toxic effects of vitamin D. The percentage bone ash levels off at vitamin D_2 doses above 400 IU/day (fig. 1, bottom) and is the same even at the higest dose of 40,000 IU/day. With vitamin D_3 the percentage values of bone ash were consistently higher than those observed with vitamin D_2, but did decrease at the highest dose of 40,000 IU/day to a value approximately that observed with vitamin D_2.

Growth, as indicated by final body weights, clearly pointed to the deleterious effects of the higher vitamin D dosages, as shown in figure 2 (bottom). These deleterious effects are also reflected in the average extracted bone weights shown

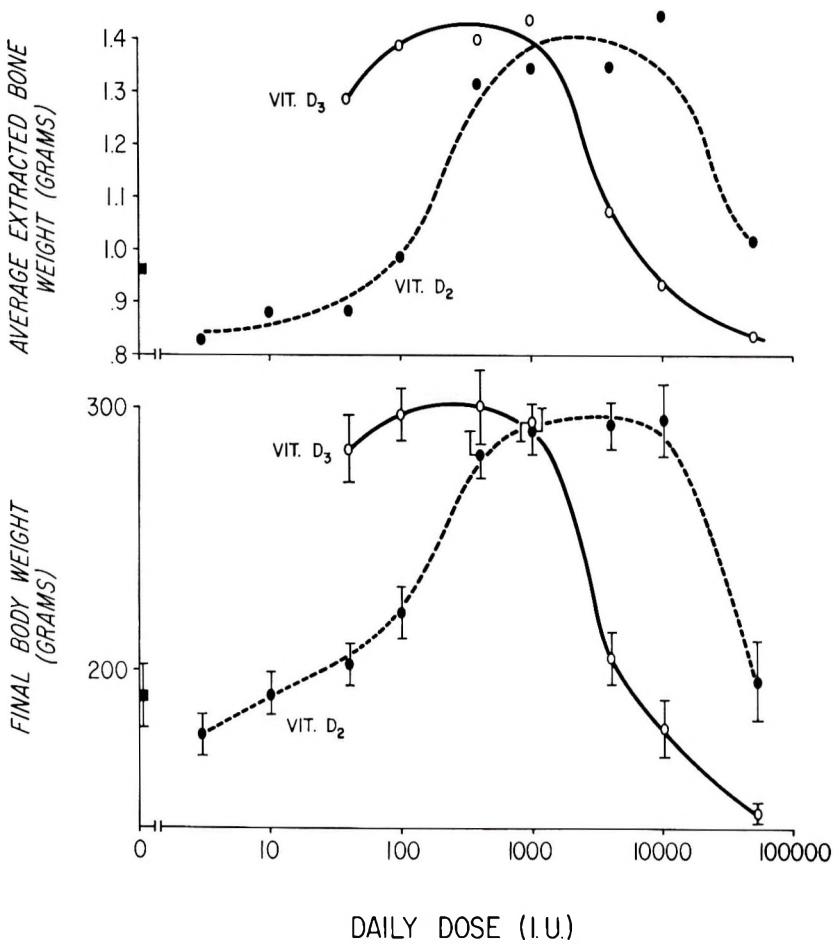

Fig. 2 Effect of vitamins D_2 and D_3 on: (top) tibia weight; and (bottom) final body weights. Body weights given as means ± SE.

in the same figure 2 (top). It appears from both of these parameters that vitamin D_3 was more potent (or toxic) than vitamin D_2 by an order of magnitude, or roughly, the same as their relative antirachitic potencies (1).

The differences noted with serum phosphorus levels also pointed to a potency ratio of about 10 (fig. 1, middle). At dosages above 10,000 IU/day vitamin D_2 and 400 IU/day vitamin D_3, the hypophosphatemic effect is clearly evident.

At the higher dosages of the D vitamins, the serum calcium levels reached a plateau of over 16 mg/100 ml and were not a variable function of the dosage given. Thus, by itself, the hypercalcemia resulting from excess doses of vitamin D appears to be a poor indicator of the overall biologic effects observed.

Differences in bone density and reactivity are evident in microradiographs and fluorescence photographs of tetracycline uptake. Under ultraviolet illumination areas of tetracycline uptake showed a golden yellow fluorescence which was in sharp contrast with the normal dull bluish "autofluorescence" of bone tissue itself.

Cortical bones from rachitic chicks demonstrated the typical microradiographic appearance shown in figure 3A, with subperiosteal rarefaction and the presence of a loose network of intra-medullary mineral. In this case the intra-medullary bone was more noticeable than in a previously published microradiograph of rachitic chick bone (1). Tetracycline was avidly deposited in the rachitic bone (fig. 3B), most heavily in the outer "periosteal" area.

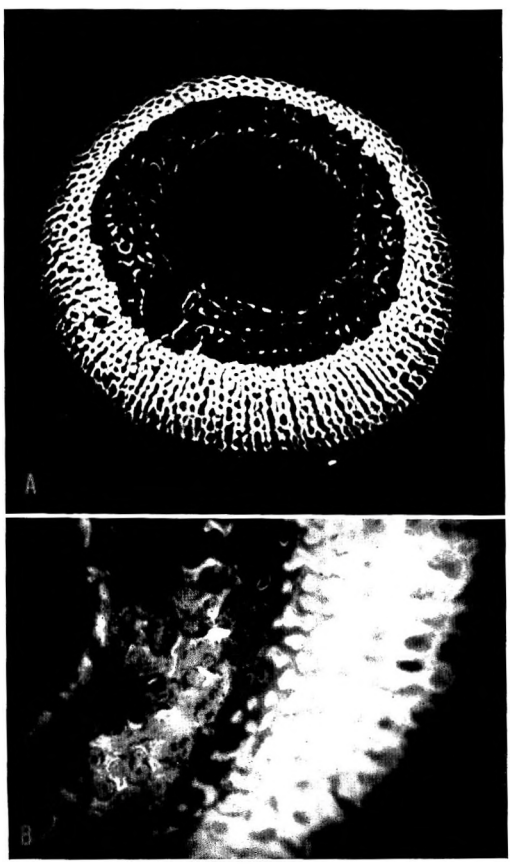

Fig. 3 Bone from rachitic control chick; A. microradiograph; and B. tetracycline uptake.

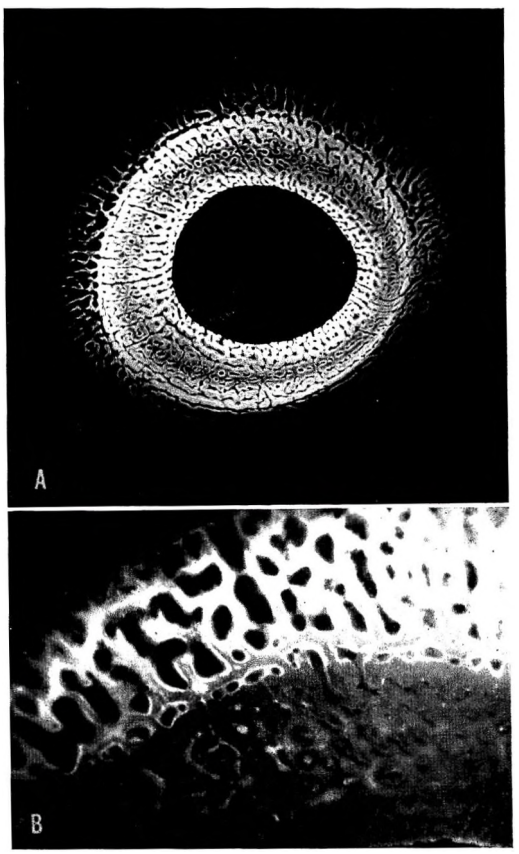

Fig. 4 Bone from chick receiving vitamin D_3, 40 IU/day; A. microradiograph; and B. tetracycline uptake.

In many of the intermediate dose groups, tetracycline deposited in a characteristic sharply defined periosteal ring. An example is the group given 40 IU/day vitamin D_3 which had the microradiographic appearance shown in figure 4A. Mineralization appeared relatively uniform across most of the shaft with rarefaction of outer cortex. However, the sharply defined endosteal surface should be noted. Tetracycline uptake by this bone showed rather sharp demarcation between an outer zone which took up tetracycline and an inner zone which did not (fig. 4B).

Even more pronounced was the definition observed at 4000 IU/day vitamin D_3, where the microradiographs showed relatively uniform mineralization all the way across the shaft (fig. 5A), whereas tetracycline uptake by the bone (fig. 5B) showed

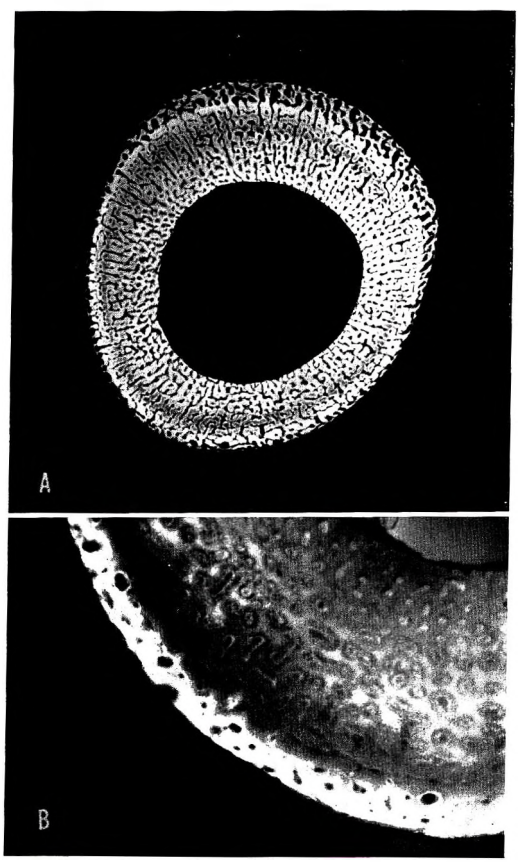

Fig. 5 Bone from chick receiving vitamin D_3, 4,000 IU/day; A. microradiograph; and B. tetracycline uptake.

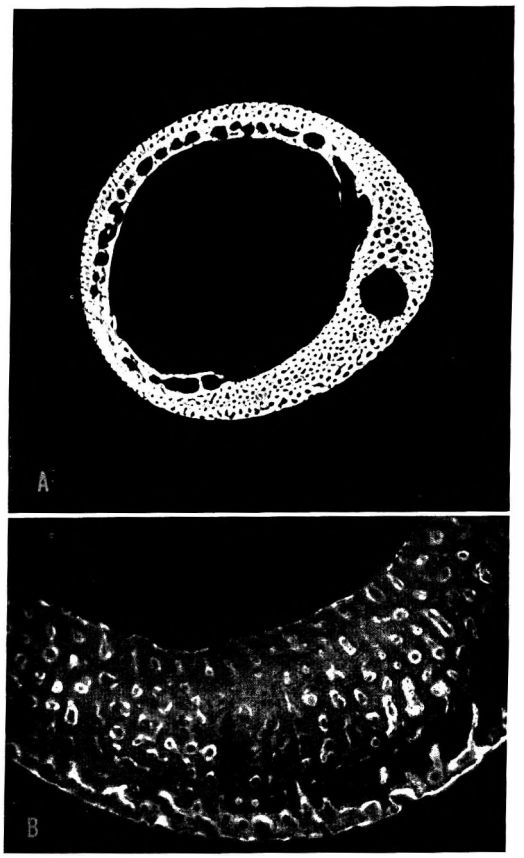

Fig. 6 Bone from chick receiving vitamin D_3, 40,000 IU/day; A. microradiograph; and B. tetracycline uptake.

that only a thin outer shell of cortical bone was capable of picking up the tetracycline.

At the highest dose level, 40,000 IU/day vitamin D_3 practically no tetracycline deposition could be observed (fig. 6B) compared with the other groups and the bone appeared extremely "punched out" with many resorption cavities and sharp endosteal and periosteal surfaces (fig. 6A).

DISCUSSION

The microradiograph of rachitic bone (fig. 3A) clearly shows pronounced subperiosteal rarefaction and also a loose network of bone within the marrow cavity. The latter observation indicates that in rickets caused by vitamin D deficiency there was impaired resorption of the interior bone simultaneous with impaired deposition of new mineral superiosteally.

In some groups, tetracycline deposited in a rather sharply defined ring, concentric to and surrounding an inner much less reactive ring. This phenomenon may be due to the fact that the vitamin D treatment was instituted after only 3 weeks of feeding the rachitogenic diet, and the differing reactivities of inner and outer cortex reflected the differences between bone laid down with, and bone laid down without, the presence of vitamin D.

In a comparative study of cod liver oil (presumably vitamin D_3) and irradiated ergosterol or vitamin D_2, Correll and Wise (2) reported that cod liver oil had little effect on serum calcium, serum phosphorus and percentage bone ash at doses between 3 and 25,000 IU/day even though 19 of the 20 birds in the latter group died and 15,000 IU/day were considered by them to be the toxic level on the basis of external appearance and growth. Furthermore, cod liver oil did not affect serum phosphorus levels as did vitamin D_2. It therefore appears that in their experiments the other constituents of cod liver oil (including possibly vitamin A) were mitigating the expected toxic effects of the vitamin D_3 present.[5]

The actions of vitamin D on both the intestine (promoting calcium absorption) and bone (promoting mineral resorption) are shown in the composite figure 2. Thus, with doses of vitamin D up to 1000 units/day, the serum calcium increased with dosage, whereas both the percentage bone ash and extracted bone weight also increased. Therefore, the increased serum calcium must have been of dietary origin.

At doses greater than 1000 units/day the serum calcium remained high but the percentage bone ash leveled off, whereas extracted bone weight decreased. From growth curves, it was observed that the chicks given high vitamin D_3 (40,000 units/day) gained weight up to 27 days and then lost weight from that time. Therefore, mineral and matrix must together have been undergoing dissolution.

The relative non-proportionality between serum calcium and dose of hypercalcemic sterol has been reported previously with studies in rats using vitamin D_2, dihydrotachysterol and Hytakerol (3).[6] A marked increase in serum phosphorus was observed, presumably due to renal failure. In contrast, chicks apparently did not develop the excessively high serum phosphorus levels but their serum calcium concentrations nevertheless leveled off with high doses of vitamin D just as observed with rats.

The basic conclusion of this study, then, is that vitamin D_3 possesses greater potency than vitamin D_2 in the chick at hypercalcemic and toxic levels. Although the serum calcium response to doses above 1000 units/day was roughly equivalent, the percentage bone ash (except for the 40,000 IU/day vitamin D_3) was consistently higher than any vitamin D_2 response.

It is difficult, however, from these parameters to assess a potency ratio between the 2 forms of vitamin D. From final body weights and the average extracted bone weights, as well as serum phosphorus responses to doses above 1000 units/day, it appears that vitamin D_3 is roughly 10 times more effective than vitamin D_2 which approximates the antirachitic "efficacy ratio" reported previously (1).

The results with tetracycline uptake were especially interesting. Tetracyclines form chelate complexes with calcium and other divalent cations (4) and when injected in vivo, deposit on sites of bone mineralization (5), largely to growing surfaces. Once buried by mineral accretion the removal of such tetracycline could not occur, but if the deposition site is inactive or resorbing (6) then removal can take place. Thus, it appears that the bones of a chick receiving 40,000 IU/day vitamin D_3 are undergoing such tremendous resorbing activity that the tetracycline either cannot bind or, if bound, is easily removed as the blood concentration of tetracycline decreases following injection. The exceptionally spongy appearance in microradio-

[5] Unpublished data, H. B. Bosmann and P. S. Chen, Jr., 1965.

[6] Marketed by Winthrop Laboratories, New York; in 1961 this was labeled as "brand of dihydrotachysterol in sesame oil"; "1 cm^3 contains the equivalent of 1.25 mg crystalline dihydrotachysterol." On the basis of pharmacological (reference 3) and physicochemical tests (Terepka, A. R., Chen, P. S., Jr. and B. Jorgensen, Endocrinology, 68: 996) it was shown that the active principle was probably dihydrovitamin D_2-II, a compound isomeric with dihydrotachysterol but which possesses only one-fifth to one-sixth of the hypercalcemic potency of the latter substance.

graphs of bones from chicks fed at the highest dose level of vitamin D_3 is consistent with such a degree of resorbing activity.

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Antagonism between Vitamins A and K in the Germfree Rat [1]

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ABSTRACT Weanling germfree male rats were fed a semi-purified diet complete except for being free of vitamin K and containing only approximately 0.4 IU of vitamin A activity/g. Vitamins A and K_1 were administered separately, vitamin A to the amount of zero, 5, 50, 200 and 2000 IU/day, and vitamin K first at a level of 2 μg/day, later to the amount of 0.5 μg/day. A daily intake of 0.5 μg/day allowed only a limited lifespan before the rats died with all the symptoms of the hemorrhagic syndrome typical of a vitamin K deficiency. Compared with the survival time observed with vitamin A intakes usually considered adequate, lifespan was shortened by a daily intake of 2000 IU/day, possibly shortened at an intake level of 200 IU, and definitely prolonged without vitamin A supplementation. This last group of rats demonstrated reduced growth associated with the low vitamin A intake, presumably leading to a lower requirement for vitamin K. It was concluded that in the usual range of vitamin A intake for germfree rats fed the diets commonly used in germfree experimentation, no effect of vitamin A intake upon vitamin K requirement is demonstrable. When vitamin A intake exceeded the optimum by a factor of 10 or more, an antagonism between vitamins A and K became apparent.

Hypervitaminosis A in the conventional rat results in hypoprothrombinemia and frequently death caused by hemorrhaging. The symptoms can be alleviated by the administration of vitamin K (1). Recently Matschiner and Doisy (2) demonstrated that the level of dietary vitamin A in the conventional rat is related closely to the development of symptoms of a vitamin K deficiency, i.e., under appropriate experimental conditions reduced prothrombin concentrations occurred when the rat received 0.5 and 5 IU of vitamin A/g of vitamin K-deficient diet, but with corresponding rations deficient in vitamin A no such depression was observed. The mechanism whereby the level of vitamin A accounts for hypoprothrombinemia and hemorrhaging when conventional rats receive adequate or suboptimal amounts of vitamin K is not known. Quick and Stefanini (3) theorized that excessive amounts of vitamin A might interfere with synthesis of vitamin K by the flora.

Nutritional studies in this laboratory on the amounts of vitamins K_1 and K_3 required by the germfree rat (4) raised the question whether a fluctuation of vitamin A levels between 300 and 700 IU/100 g diet due to diet sterilization and subsequent storage in germfree isolators, had influenced our results. Experiments using germfree rats were designed to answer the above question and to determine whether results similar to those of Matschiner and Doisy (2) could be obtained in the absence of microbial flora, ruling out an intestinal flora alteration as the cause of hypoprothrombinemia.

MATERIALS AND METHODS

Lobund-strain germfree weanling male rats of Wistar origin were used in all experiments. One week prior to weaning, the litters were fed diet L-467E-15,[3] a semi-purified diet (table 1) complete except for being deficient in vitamin K and containing approximately 0.4 IU vitamin A activity/g (0.1 IU as vitamin A plus 0.3 IU as carotene).[4] The diet was steam sterilized for 25 minutes at 123°. Weanling males were placed in stainless steel screen cages within the Reyniers system, and provided with filter paper bedding. Diet

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[1] These studies were supported by a grant from the Nutrition Foundation and by general support from the National Institutes of Health and from the University of Notre Dame.
[2] St. Mary's College, Notre Dame, Indiana.
[3] Diet used in initial experiment (results in fig. 1) contained vitamin-free casein extracted in this laboratory.
[4] Chemically analyzed as outlined by D. B. Parrish, Report of Vitamin A in Mixed Feeds, J. Assoc. Off. Agr. Chemists, *41*: 593, 1958.

TABLE 1

Composition of semi-purified diet L–467E–15

	%		%
Casein, extracted [1]	24.0	*i*-Inositol	0.1
Peanut oil	5.0	Vitamin B mix 75R	0.5
Lad(ek)–48 [2]	2.0	Minerals L–2	5.0
DL-Methionine	0.4	Powdered cellulose [3]	5.0
dl-α-Tocopherol	0.01	Rice starch, extracted	58.0
	g		*mg*
Minerals L–2		Vitamin B mix-75R	
$CaCO_3$	1.5	Thiamine	6.0
$CaHPO_4$	0.275	Riboflavin	3.0
K_2HPO_4	1.125	Nicotinamide	5.0
Na_2HPO_4	1.0	Nicotinic acid	5.0
NaCl	0.25	Ca pantothenate	30.0
KI	0.00375	Choline chloride	200.0
$MgSO_4 \cdot H_2O$	0.375	Pyridoxine·HCl	2.0
$MnSO_4 \cdot H_2O$	0.0625	Pyridoxamine dihydrochloride	0.4
$Fe(C_6H_5O_7)_2$	0.375	Biotin	0.1
$CuSO_4$	0.0192	Folic acid	1.0
$CoCl_2 \cdot 6H_2O$	0.0025	*p*-aminobenzoic acid	5.0
$ZnSO_4 \cdot H_2O$	0.005	0.1 Trituration vitamin B_{12}	
$Na_2B_4O_7 \cdot 10H_2O$	0.0025	in mannitol	25.0
$AlK(SO_4)_2 \cdot 12H_2O$	0.00375	Extracted rice starch carrier	217.5
		Lad(ek)-48 [2]	
		Vitamin A (acetate) [4]	0.274
		Vitamin D (calciferol)	0.0025
		Peanut oil to 2.0 g	

[1] Labco, The Borden Company, New York.
[2] Lad(ek) is a term coined by this laboratory to designate fat-soluble vitamin mixtures.
[3] Cellophane Spangles, Microfibre, Inc., Pawtucket, Rhode Island.
[4] Omitted in these studies.

L-467E-15 and water were available ad libitum. Limited production of germfree male rats necessitated bringing various groups of animals into the system over a period of several weeks. This accounts for differences in starting time in the first experimental series, the combined data of which are shown in figure 1.

Aliquots of crystalline vitamin A acetate [5] and vitamin K_1 [6] were dissolved in peanut oil. The peanut oil itself was virtually devoid of vitamin A, containing on the order of 0.2 μg of carotene/g [7] (presumably corresponding to approximately 0.3 IU vitamin A) and was free of vitamin K (4). The mixtures were filtered through a G. S. Millipore filter (0.22-μ pore size) with a glass fiber prefilter. The flasks of oil were sealed in a flame, sterilized in the entry port of the isolator for 20 minutes with 2% peracetic acid containing 0.1% Nacconal [8] and then taken inside the Reyniers system.

In the first preliminary series the weanling male rats were divided into 4 groups, each group being housed in a separate wire cage. Animals of each group were fed by tuberculin syringe 0.2 ml of the vitamin A and K_1 supplement in peanut oil per day. The supplement of vitamin A amounted to 5, 50, 200 and 2000 IU/day for the respective 4 groups (referred to hereafter as 5A, 50A, 200A and 2000A). The amount of vitamin K_1 was initially 2 μg daily. As the experiment proceeded this was reduced to 1.0, 0.5 and finally to zero micrograms per day (fig. 1). The progressive decrease in vitamin K_1 was necessary since at even the highest levels of vitamin A hemorrhagic death rarely occurred when 2 μg vitamin K_1 were given daily.

In 2 subsequent series each animal received a daily supplement of 0.5 μg vitamin K_1 and zero, 5, 50, 200 or 2000 IU of vitamin A. In the last experimental series, designed to study the gain in body weight of germfree rats fed diets containing low

[5] Nutritional Biochemicals Corporation, Cleveland.
[6] See footnote 5.
[7] Fleischmann Laboratories, Stamford, Connecticut, personal communication.
[8] Sodium alkylarylsulfonate, obtained from Fisher Scientific Company, Chicago.

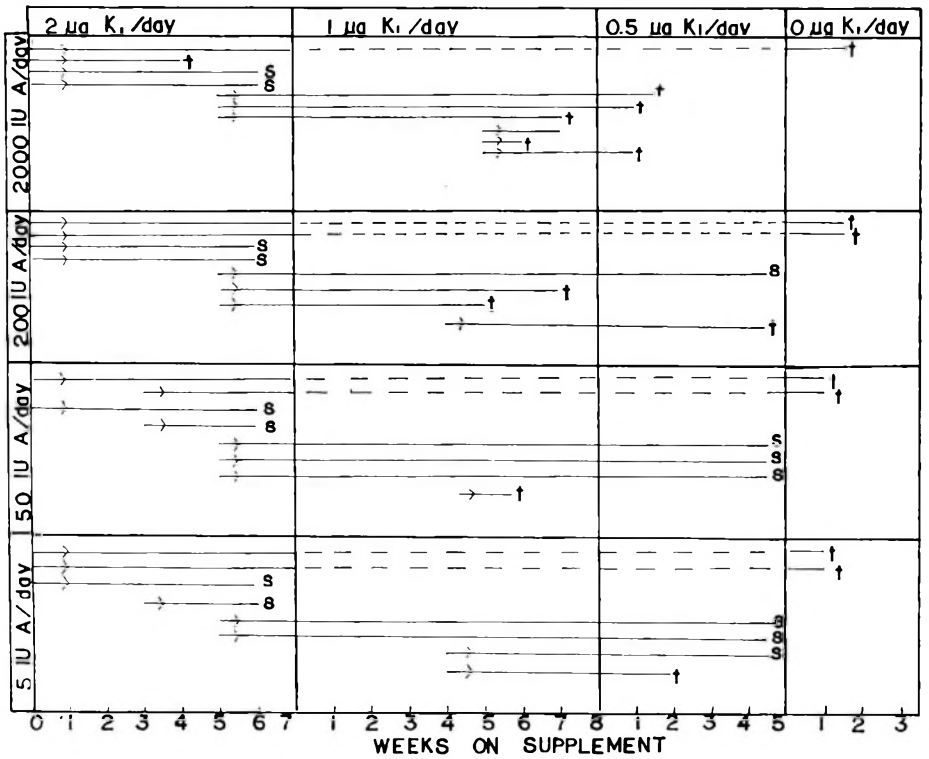

Fig. 1 Effect of vitamin A and vitamin K supplementation on survival in the male weanling germfree rat fed vitamin K-free diet L-467E-15. Key:——— life span of animal fed specific vitamin A supplement and given decreasing supplements of vitamin K_1 as indicated; – – – – –, life span of animal transferred directly from 2 μg vitamin K_1/day supplement to vitamin K_1-unsupplemented diet. Animals entered the various vitamin K supplement groups at different times in the experiment (see text).

levels of vitamin A, daily supplements of 0.5 μg vitamin K_1 and zero, 5, or 50 IU of vitamin A were given.

In all experiments survival time or hemorrhagic death was a main criterion. Whenever animals were killed, prothrombin times were determined by the method of Quick (5).

RESULTS AND DISCUSSION

The first series was designed to obtain a qualitative notion of the relationship between vitamin A and vitamin K_1 in germfree rats, and to establish the amount of vitamin K_1 which had to be incorporated in the diet to gauge the effect of vitamin A in a concentration range which could be expected in these diets after sterilization and storage. Figure 1 shows the results of this preliminary series in which 34 weanling germfree male rats were used. Not all animals were available at the same time, thus accounting for the different times of entry at the various levels of K supplementation. Twenty-seven animals, divided into 4 groups representing a supplemental vitamin A intake of 2000, 200, 50 and 5 IU/day, were started with a vitamin K_1 supplement of 2 μg/day. Eleven of these animals were given this supplement for 2 weeks, after which time the amount of vitamin K_1 was reduced to 1 μg and then to 0.5 μg daily. Of the 16 remaining rats receiving the 2 μg/day supplement, eight were killed for prothrombin time after 3 to 6 weeks, one died because of hemorrhaging, and the other animals were transferred after 7 weeks directly to the unsupplemented vitamin K_1-free diet (indicated by broken lines in fig. 1). Seven animals were started with a 1-μg vitamin K_1 daily supplement. For four of these,

the supplement was reduced to 0.5 μg after 3 weeks.

Animals started with a supplement of 2 μg vitamin K_1/day survived for at least 6 weeks at all levels of vitamin A supplementation, except in the 2000 A group, where one out of three animals died after 4 weeks. When the vitamin K_1 supplement was discontinued, all animals fed at all vitamin A levels died within one to two weeks.

Animals in groups 5A and 50A which were started with 2 μg vitamin K_1 daily and then changed to 1 μg after 2 weeks, survived for 8 weeks. However in group 200A, two out of three and in group 2000A one out of three died during that period. The survivors that were given 0.5 μg vitamin K_1 lived for 5 weeks in groups 5, 50 and 200A, but in group 2000A all died in less than 2 weeks.

Seven animals were started with the marginal amount of 1 μg vitamin K_1/day. The 2 in group 5A survived for 4 weeks and one animal subsequently survived a change to 0.5 μg vitamin K_1 for several weeks before being killed, whereas the other animal died of unknown causes after receiving this supplement for 2 weeks. The animals in the group receiving 50, 200 or 2000 IU vitamin A died within 4 weeks after receiving the 1-μg vitamin K_1 supplement or upon a change to the 0.5-μg vitamin K_1 supplement.

All deaths, except the one aforementioned, were caused by massive hemorrhaging into the subdural space, into the thoracic or abdominal cavities, around the epididymis, or subcutaneously. The animals that were killed in the 4 vitamin A supplementation groups while receiving a 2-μg vitamin K_1 daily supplement, showed normal prothrombin times (average, 25 seconds). Those killed while receiving a 0.5-μg vitamin K_1 and a supplement of 5, 50 or 200 IU vitamin A/day showed prothrombin times greater than 1 minute.

From this preliminary experiment the following conclusions were drawn: (a) A daily vitamin K_1 intake of 0.5 μg to 1 μg provides the most critical test for the effect of a vitamin A intake in the range of 5 to 2000 IU/day. (b) Even after 8 weeks of a daily intake of 2 μg vitamin K_1, depletion of vitamin K_1 in germfree rats is rapid after omission of the supplement. (c) Upon supplementation of the diet with 1 μg and later 0.5 μg of vitamin K_1, death resulting from hemorrhaging was zero out of three animals in the 5A group, one out of four in the 50A group, three out of four in the 200A group, and six out of six in the 2000A group, thus indicating an effect of vitamin A intake upon vitamin K_1 requirements.

Since the use of a vitamin K-free diet supplemented by 0.5 μg vitamin K_1/day appeared to provide a most sensitive experimental system to test the effect of vitamin A intake, 2 experiments were carried out using various amounts of vitamin A and only 0.5 μg vitamin K_1 daily in the supplement. In all, 47 animals were used. A commercially extracted casein [9] was incorporated in the diet. The results of the first series showed that this casein had some residual vitamin K_1 activity, as demonstrated by a combined average survival time of 45.8 days for all vitamin A levels. Improved casein extraction procedures adopted by the manufacturer lowered the average survival time in the second series to 25.3 days, a value more consistent with that obtained using the casein extracted in the laboratory (4). To compare the 2 experiments, survival times at the various vitamin A levels within each series were expressed on the basis of the combined average survival time of that series (table 2). The results indicate that in both series the survival times with no vitamin A supplement and the survival times with 2000 IU/day were significantly different from the narrow range of values obtained with supplementation ranging from 5 to 200 IU/day. The unsupplemented groups representing an intake equivalent to approximately 5 IU of vitamin A/day showed an increase in survival time and therefore indicated a lower vitamin K_1 requirement. The 2000 IU group definitely showed shorter survival, thereby indicating an increased requirement for vitamin K_1. Matschiner and Doisy (2) had also indicated that rats had lower vitamin K requirements when fed a vitamin A-deficient diet. It occurred to us that these animals might show a reduced requirement because of

[9] See footnote 5.

impaired growth on the essentially vitamin A-deficient diet. To test this hypothesis 30 weanling male germfree rats were divided into 3 groups as follows: One group (9 animals) received no vitamin A in the supplement, the second group (11 animals) received 5 IU vitamin A/day and the third group (10 animals) received 50 IU vitamin A/day. Animals of all 3 groups received 0.5 μg vitamin K_1 daily. Results (fig. 2) indicate that on the twenty-eighth day animals of the 5A and 50A groups were generally of the same weight, whereas those in the zero A group weighed slightly less. On the fifty-sixth day animals in the 50A showed better growth than those in the 5A, and the latter showed better growth than those in the zero A group. Seventeen of the original 30 animals died because of hemorrhaging during the first 35 days of receiving the supplement (a greater than 50% mortality). Two other animals, both in the 50A group, died between the thirty-fifth and fifty-sixth day. Individual growth curves of these 19 animals showed no loss of weight before death, and furthermore indicated that they were not in a lower weight range, confirming data obtained earlier in this laboratory (4). The pattern of death and the pro-

TABLE 2

Survival of germfree rats fed semi-purified diet L-467E-15 with various amounts of vitamin A acetate and 0.5 μg of vitamin K_1 administered daily

Experiment no.	27A	28A	
No. animals	23	24	
Avg survival time (days) total groups ($\bar{s}$) [1]	45.8	25.3	
Vitamin A acetate supplement subgroup	Avg survival (days) subgroups [2] / Avg survival (days) total groups ($\bar{s}$)		Avg
IU/day			
0	1.24	1.42	1.33
5	0.97	1.02	1.00
50	1.04	0.88	0.96
200	0.96	0.98	0.97
2000	0.79	0.70	0.74

[1] $\bar{s}$ = combined average survival of all 5 dietary groups (see text).
[2] Four to five animals/subgroup.

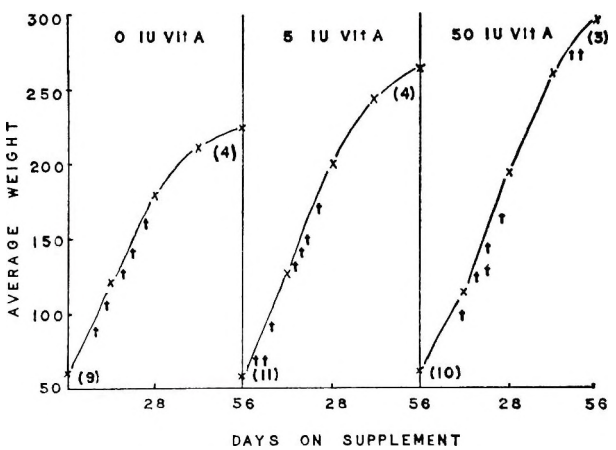

Fig. 2 Average growth of male weanling germfree rats fed diet L-467E-15 plus various amounts of vitamin A acetate and 0.5 μg vitamin K_1 added daily. Key: ( ), number of animals at start and after 56 days; +, animals died.

longed prothrombin times (96 to 154 seconds) of the survivors of all 3 groups were generally comparable.

These data show that with a vitamin A intake which is generally considered subnormal, the growth of the germfree rat is reduced, and suggest that a lower daily requirement for vitamin K is caused by the retarded growth of these young rats and results in longer survival when fed at a submarginal vitamin K intake. According to the National Research Council (6) the vitamin A requirement for the growing rat is 200 IU/100 g diet or approximately 20 IU/day (6). The intake with the unsupplemented purified diet falls far below this level, and even in the 5A group the calculated total intake is slightly lower than 10 IU/day. Therefore only the reduction in survival time as shown in the preliminary series in the 200 and 2000A groups and in the later experiments only in the 2000A group, can be considered as truly indicative of an antagonism between the vitamins A and K. Obviously, as this antagonism is demonstrable in the absence of a potentially vitamin K-producing microflora, it must occur on the level of the systemic metabolism.

These data do not confirm the results of Matschiner and Doisy (2) in the lower vitamin A range, but they do indicate an antagonism between vitamin A and K when the intake of vitamin A/day is 2000 IU (and possibly 200 IU). The fact that larger doses of vitamin A (2000 IU/day) antagonize the action of vitamin K_1 in germfree rats refutes the opinion of Quick and Stefanini (3), who speculated that these doses interfere with the microbial production of K vitamins in the intestine. The data furthermore, show that changes in vitamin A intake occurring in this laboratory's nutritional studies (4) lie within a safe range and thus would not have influenced our results.

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Metabolic Response to Realimentation Following Chronic Starvation in the Adult Male Rat [1]

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ABSTRACT Changes in liver weight, liver glycogen and lipid concentrations, and certain hepatic enzyme activities were studied during realimentation of adult male rats previously starved 9 to 12 days. Liver weight increased nearly twofold during the first 48 hours of refeeding and then declined to normal at 96 hours post-refeeding. Composition of the hepatic tissue showed similar marked changes. Glycogen concentration increased rapidly to a maximum of 157 mg/g fresh liver at 24 hours post-refeeding, then declined to 48 mg/g at 72 hours post-refeeding. Lipid concentration which increased during starvation decreased rapidly during the first 12 hours of refeeding, then increased to 69 to 88 mg/g liver at 72 to 96 hours after refeeding. Assays for glucokinase, isocitrate dehydrogenase (NADP-linked) and glucose-6-phosphate dehydrogenase revealed differential responses in activity during realimentation. The response in G-6-P-DH activity, however, was markedly affected by the protein content of the refeeding diet.

Hyperlipogenesis is a frequently reported response to realimentation following food deprivation. Longenecker (1) reported that when starved adult rats were refed either high carbohydrate or high protein diets one-third of the total weight gain was deposited as fat. Tepperman and Tepperman (2) observed that, although liver *de novo* fat synthesis declined to near zero during a 48-hour fast, within 24 hours after the beginning of refeeding lipogenic activity increased to 800% of the rate observed in non-starved controls. Similar changes in lipogenesis have been observed in the starved-refed growing chicken (3).

Other adaptive changes also occur during realimentation. One response that has been reported is the marked increase in hexose monophosphate shunt activity (4). Glucose-6-phosphate dehydrogenase (G-6-P-DH) in the livers of fasted rats has been observed to increase to a peak activity, several times the level of that in non-starved controls at 72 hours after the start of refeeding (5). Demonstration by Potter and Ono (5) that puromycin would prevent the "overshoot" in G-6-P-DH activity in refed rats indicates that the increased activity during refeeding is the result of *de novo* synthesis of apoenzyme protein. These authors also observed that the proportion of carbohydrate and protein in the refeeding diet had a marked modifying effect on the dehydrogenase activity.

Most observations on the metabolic transition accompanying realimentation have been conducted with animals fasted for only 48 to 72 hours. Because investigations in our laboratory have been concerned with the response to refeeding following severe starvation (6, 7), a study was undertaken to follow the changes in metabolism associated with refeeding following chronic starvation. Included in the study was an investigation of the effect of diet composition on the metabolic transition observed during realimentation.

METHODS AND MATERIALS

Adult male rats weighing 400 to 500 g were maintained in separate, wire-floor cages and fed ad libitum the following diet: (in %) isolated soy protein, 18.0; glucose,[3] 74.8; cottonseed oil, 2.0; salts 4164 (8), 4.0; vitamin mixture in glucose,[4]

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[4] See footnote 3.

0.5; choline chloride, 0.1; DL-methionine, 0.6; and α-tocopheryl succinate, 0.025. Vitamins A and D were administered in the diet to provide 250 and 60 IU/day, respectively. The vitamin premix contained per 100 g: (in milligrams) thiamine chloride·HCl, 200; riboflavin, 100; nicotinic acid, 500; pyridoxine·HCl, 100; Ca pantothenate, 200; menadione, 20; folic acid, 20; biotin, 2; and vitamin B_{12} (as 0.1% triturate in manitol), 2. During experimental periods the animals were starved until they lost approximately 20% of their prestarvation body weight (9 to 12 days' starvation) following which they were refed ad libitum for varying periods from zero to 96 hours. Drinking water was provided at all times.

Following the designated refeeding period the rats were decapitated. The livers were excised rapidly, blotted on filter paper and weighed. A sample (2 to 3 g) of each liver was placed in 5 volumes of cold homogenizing buffer,[5] minced with scissors and homogenized with a Potter-Elvehjem homogenizer. The remainder of each liver was immediately frozen and stored for later total lipid analysis. In experiment 1, an aliquot of the homogenate was extracted with 30% KOH and analyzed for glycogen by the anthrone method (9) using glycogen [6] as the standard. The remaining homogenate was centrifuged 20 minutes at 26,000 × *g* in a Servall Model SS1 centrifuge. The G-6-P-DH (+ 6-phosphogluconic dehydrogenase) [7] activity was assayed in the resulting supernatant by the method of Lohr and Waller (12). In experiments 2 and 3, the homogenate was centrifuged 10 minutes at 2400 × *g* and 20 minutes at 11,000 × *g* in the Servall centrifuge, and 60 minutes at 105,000 × g in Spinco Model L ultracentrifuge. Glucokinase, G-6-P-DH[8] and isocitrate dehydrogenase (NADP-linked) activities were determined in the high-speed supernatant fraction. Glucokinase activity was measured by following the appearance of NADPH in the presence of purified G-6-P-DH (10, 11).[9] The G-6-P-DH activity was assayed according to Lohr and Waller (12), and isocitrate dehydrogenase activity was determined by the method of Plaut (13). All measurements were carried out at 37° with a Perkin-Elmer Model 202 recording spectrophotometer equipped with a time rate accessory. The amount of NADPH formed was calculated from the change in optical density using the extinction coefficient 6.22×10^6 cm^2/mole for NADPH (14). The disodium salt of ATP, monosodium salt of NADP, disodium salt of G-6-P and trisodium citrate used in the assays were obtained commercially [10] and used as supplied by the manufacturer.

Lipid content of the livers was determined gravimetrically by the technique of Sperry (15). The liver was disintegrated with a Potter-Elvehjem homogenizer provided with a loose-fitting Teflon pestle, dehydrated with acetone and extracted with chloroform-methanol (2:1, v/v). Protein was estimated by the procedure of Lowry et al. (16), using Folin and Ciocalteau reagent purchased commercially [11] and crystalline bovine plasma albumin [12] as standard.

RESULTS

When adult rats were starved for 9 to 12 days there was a proportionally greater loss in liver weight than in the body weight (figs. 1 and 2). During the first 24 to 48 hours of realimentation, however, liver weight changed much more rapidly than body weight as evidenced by the significant increase in liver-to-body weight ratios. In the series of experiments reported here, average liver weight decreased from 12 to 14 g in the non-starved control animals to 7 to 9 g in animals starved for 9 to 12 days. On refeeding, average liver weight increased to a maximum of 15 to 17 g at 48 to 72 hours after the beginning of refeeding and then decreased sharply to 12.5 to 14.5 g at 96 hours post-refeeding. However, body weight continued to increase over the entire refeeding period. As a consequence, liver-to-body weight ratio is a function of the coincident change in liver and body weight during starvation and refeeding.

[5] Exp. 1, EDTA-physiological saline (13); exps. 2 and 3, buffer of Sharma et al. (11).
[6] Fisher Scientific Company, Chicago.
[7] In view of the results of Potter and Ono (5) no attempt was made to distinguish between G-6-P-DH- and 6-phosphogluconic dehydrogenase activities and it was assumed that the differences noted were primarily attributable to G-6-P-DH activity.
[8] See footnote 7.
[9] See footnote 7.
[10] Sigma Chemical Company, St. Louis.
[11] See footnote 6.
[12] See footnote 10.

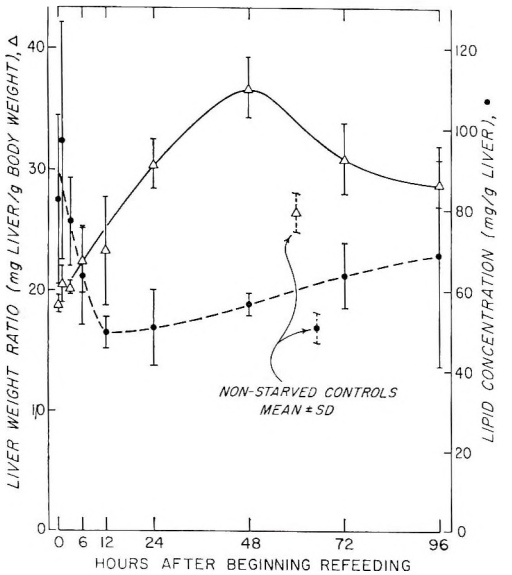

Fig. 1 Effect of refeeding following starvation, on liver weight as related to body weight and on liver lipid concentration (exp. 1) Key: Liver weight change (△———△) and liver lipid concentration (●- - - - -●) during refeeding following starvation. Each point in these and all subsequent figures represents the mean of determinations on 3 rats, while the vertical line through each point indicates the standard deviation of the mean.

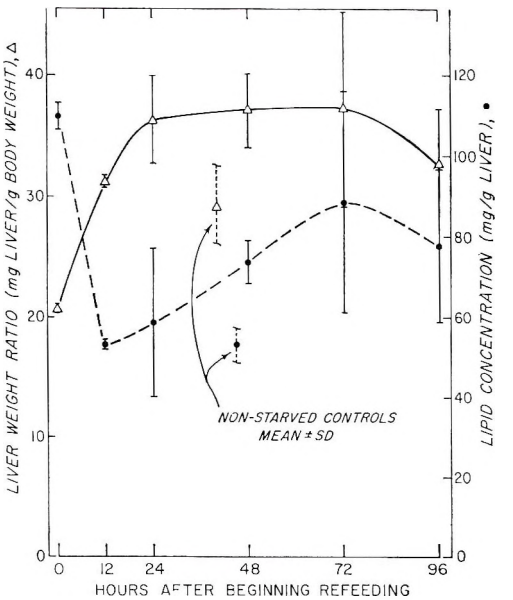

Fig. 2 Effect of refeeding following starvation, on liver weight as related to body weight and on liver lipid concentration (exp. 2). Key: as in figure 1.

The marked changes that occurred in liver weight in the course of realimentation of starved rats were accompanied by equally striking changes in the composition of this organ. The concentration of liver lipid, which increased considerably during starvation, decreased rapidly during the first 12 hours of refeeding (figs. 1 and 2). Although much of the early decline in lipid concentration probably represented a simple dilution phenomenon, the entire change during the first 12 hours of refeeding could not be accounted for by changes in liver weight alone. Calculation of the lipid content per liver in starved rats and in animals refed for 12 hours indicated that the total lipid content actually decreased during the early refeeding period (0.9 vs. 0.44 g/liver, exp. 1; 0.92 vs. 0.72 g/liver, exp. 2). Following the initial decrease in lipid concentrations there was an increase in the proportion of chloroform-methanol extractable material. This moderate increase in lipid concentration together with the increase in liver size resulted in a considerable increase in the total lipid content of the livers at 72 hours post-refeeding. The changes in liver weight and lipid concentration in these experiments with adult rats, however, were much less marked than those reported by Smith and Johnson (6) and Soberon and Sanchez (17) with young growing animals.

Two of the most notable responses to realimentation, the large increase in liver glycogen content and the marked stimulation of hepatic G-6-P-DH activity are shown in figure 3. Refeeding resulted in an immediate response in liver glycogen synthesis. During the first 24 hours of refeeding liver glycogen increased linearly to a level 12 times that observed in starved animals and 3 times that of non-starved controls. Although there was a remarkable accumulation of liver glycogen early in refeeding, the rapid decline in glycogen concentration during the subsequent 48 hours resulted in levels similar to those of the non-starved animals at 72 hours post-refeeding. The changes in glycogen concentration were matched by equally

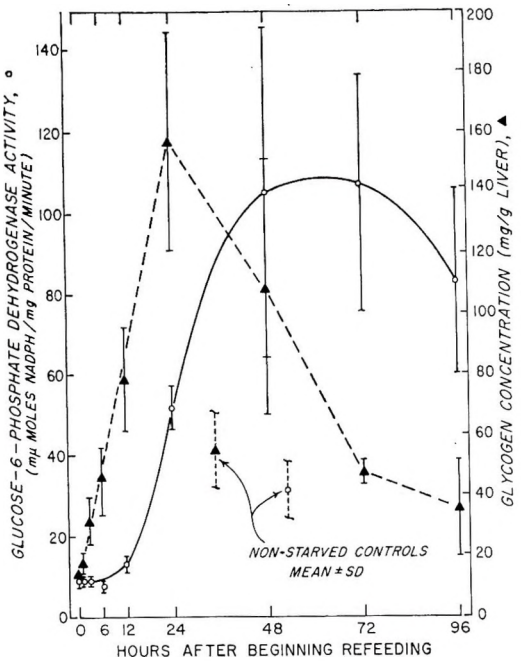

Fig. 3 Effect of refeeding following starvation, on liver glucose-6-phosphate dehydrogenase concentration and on liver glycogen concentration (exp. 1) Key: Glycogen (▲- - - - -▲) and glucose-6-phosphate dehydrogenase (○———○) values during a 96-hour refeeding period following chronic starvation.

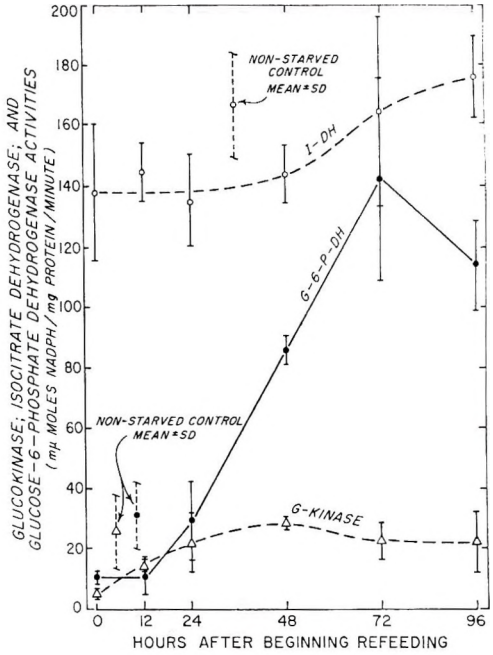

Fig. 4 Effect of refeeding following starvation, on liver glucokinase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase concentration (exp. 2). Key: Glucokinase (△- - - -△), isocitrate dehydrogenase (○- - - -○), and glucose-6-phosphate dehydrogenase (●———●) activity in starved-refed rats.

marked changes in G-6-P-DH activity (figs. 3 and 4). In both experiments, G-6-P-DH activity decreased during starvation to a level approximately 30% of that observed in the non-starved controls. Unlike glycogen synthesis, dehydrogenase activity showed no change during the first 12 hours following the resumption of refeeding. After this initial lag period, however, G-6-P-DH activity increased sharply to a maximum at 48 to 72 hours and then decreased slightly by 96 hours. The pattern of the refeeding response for glycogen metabolism and G-6-P-DH activity observed in these studies with rats starved 9 to 12 days was similar to that reported by Potter and Ono (5) with rats fasted for 72 hours, although the magnitude of "overshoot" in dehydrogenase activity was considerably less in the present study. In addition to G-6-P-DH, experiment 2 (fig. 4) included assays for glucokinase, another important enzyme of glucose metabolism, and isocitrate dehydrogenase (NADP-linked), another enzyme catalyzing the reduction of nicotinamide adenine dinucleotide. The activity of both of these enzymes, like G-6-P-DH, decreased during starvation but, unlike the latter dehydrogenase, neither glucokinase nor isocitrate dehydrogenase showed "overshoot" in activity during refeeding. However, there was a difference in the pattern of response with these 2 enzymes. On refeeding, glucokinase activity increased without any apparent lag period, whereas isocitrate dehydrogenase showed a prolonged lag in activity. Potter and Ono (5) and Blumenthal et al. (18) also reported an immediate response in glucokinase activity upon refeeding rats previously fasted for 48 to 72 hours.

Diet markedly influences the metabolic response accompanying realimentation following fasting (3, 5, 18, 19); thus varying the composition of the diet should provide a means of determining which nutrient was responsible for the induction and

TABLE 1

Responses accompanying realimentation of starved rats

Treatment group	Liver wt	Liver lipid conc	G-6-P-DH	Glucokinase activity	Isocitrate dehyd. activity
	g	*mg/g*	*mμmole NADPH/mg protein/min*		
Non-starved controls	14.4 ± 2.0 [1]	52.5 ± 4.2	31.0 ± 4.0	25.7 ± 12.2	166 ± 17
Starved	8.9 ± 1.4	104.1 ± 8.1	10.5 ± 1.8	5.0 ± 1.3	138 ± 23
Starved-refed 72 hr (18% soy protein diet)	17.1 ± 2.5	88.2 ± 27.4	142.0 ± 33.4	22.8 ± 5.8	164 ± 31
Starved-refed 72 hr (4% protein) [2]	18.9 ± 0.8	83.3 ± 10.0	33.1 ± 3.7	19.8 ± 2.8	103 ± 33

[1] Mean of 3 animals ± SD.

[2] Composition of 4% protein diet: (in %) dried egg white, 5.0; glucose (Cerelose, Corn Products Co., Argo, Ill.), 45.0; cornstarch, 38.4; cottonseed oil, 5.0; cellulose (Solka Floc, Brown Co., Berlin, N. H.), 2.0; salts 4168 (8), 4.0; vitamin mixture in glucose, 0.5; and choline chloride, 0.1.

"overshoot" of G-6-P-DH. The results in table 1 show that refeeding a very low protein diet greatly depressed the response of G-6-P-DH, but had no effect on the apparent rate of hepatic lipogenesis, suggesting that hexose monophosphate shunt activity is not necessarily associated with accumulation of liver lipid in the starved-refed rat. Glucokinase and isocitrate dehydrogenase similarly showed independent responses to changes in the protein level of the refeeding diet.

In view of the response shown in table 1, a further experiment was carried out to determine whether the induction of G-6-P-DH was due, in fact, to the protein part of the diet. Rats were fed the 4% egg protein diet (i.e., protein-starved, but not energy-starved), for 12 to 14 days, and this resulted in the same decrease in liver G-6-P-DH as did complete starvation (table 2). However, refeeding protein did not result in rapid induction and "overshoot" of the enzyme after 72 hours.

DISCUSSION

The results here reported show that the metabolic responses accompanying refeeding following 9 to 12 days of complete food deprivation are similar to those reported previously for refeeding following a 48 to 72 hour fast (2, 5). During realimentation both liver glycogen concentration and G-6-P-DH activity showed transitory increases to levels several times those observed in the non-starved control animal. Glucokinase and isocitrate dehydrogenase (NADP-linked) activities, however, showed only moderate increases to levels equivalent to those of the non-starved control. The pattern of response for the respective systems, however, was very different (figs. 3, 4). Liver glycogen showed an immediate and linear increase in concentration. Glucokinase activty, also, immediately responded to refeeding stimulus. However, G-6-P-DH activity showed an initial 12-hour lag in response and did not reach maximal activity until 48 to 72 hours post-refeeding. That puromycin prevents the recovery of glucokinase activity (10) or the "overshoot" in G-6-P-DH activity (5) suggests that the recovery of these enzyme systems represents synthesis of new enzyme protein. Whether the 12-hour lag in G-6-P-DH response is the result of a lack of proper stimulus to initiate synthesis of this enzyme protein, or the lack of specific messenger-RNA to direct enzyme synthesis, requires further investigation. The present data, however, emphasize the importance of measuring the biochemical changes associated with realimentation at enough intermediate points to establish the pattern and magnitude of the various responses accompanying metabolic transition.

One of the more interesting aspects of the present experiments was the demonstration that the pattern of response for any particular biochemical reaction may depend to a large extent on the composi-

TABLE 2

Response to protein of protein-depleted rats

Treatment group	G-6-P-DH
	mμmole NADPH/ mg protein/min
Protein-starved [1]	8.5
Protein-starved and refed 18% soy protein diet for 72 hr	9.4
Normal non-starved rats	24.8

[1] Adult rats fed the 4% egg protein diet for 12 to 14 days.

tion of the diet offered. At 72 hours post-refeeding animals fed a diet containing 4% protein had liver G-6-P-DH activities similar to those of the non-starved controls, whereas rats receiving a diet containing 18% soy protein had activities of over 4 times those of the non-starved controls. Despite the marked effect on dehydrogenase activity, changing the composition of the diet had no effect on apparent hepatic lipogenesis (table 1). The lack of association between lipid accumulation and G-6-P-DH activity suggests that liver hexose monophosphate shunt activity is not necessarily associated with increased lipogenic activity in the starved-refed rat. A similar lack of association between these 2 enzyme systems has been reported in fatty livers induced by threonine deficiency (20).

Both total starvation (table 1) and protein starvation with adequate energy intake (table 2) resulted in marked depression of liver G-6-P-DH. Induction and "overshoot" in level of this enzyme occurred only when the complete ration was refed following total starvation. This appears to indicate that the inducer is the carbohydrate of the refed diet, but also that an adequate level of dietary protein is required to replenish the amino acid pool to a level which will allow this rate of enzyme synthesis.

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Effects of Non-essential Fatty Acids on Essential Fatty Acid Deficiency [1]

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ABSTRACT The effects of the methyl esters of fatty acids of chain length C_1 through C_{18}, including oleic, elaidic, and linoleic, on essential fatty acid deficiency were studied in feeding tests with rats. Using depressed growth response as a criterion, all of the fatty acid esters except oleate and linoleate accentuated essential fatty acid deficiency, with the esters of fatty acids C_4 through C_{10} and elaidate exhibiting a much greater effect than the longer-chain fatty acid esters. The highest mortality was observed in females fed caproate, caprylate and laurate and in males fed caprylate, laurate and stearate. When reproductive performance is judged, methyl esters of myristate, palmitate, stearate, oleate and linoleate improved the reproductive performance of female rats fed the fat-free diets and, therefore, partially alleviated the essential fatty acid deficiency. Where cholesterol levels are used as criteria, supplementation with laurate and oleate resulted in an increase in the accumulation of hepatic cholesterol esters over that obtained when an unsupplemented fat-free diet was fed, indicating that laurate and oleate accentuate essential fatty acid deficiency in this respect. It is concluded that several metabolic pathways and physiological responses affected by essential fatty acid deficiency can be influenced by the concomitant presence of non-essential fatty acids. It is further concluded that evaluation of essential fatty acids status should involve more than one measurement.

The importance of essential fatty acids in animal nutrition is well documented (1). The necessity for dietary essential fatty acids to provide a healthy skin and normal internal organ function and to support and regulate growth, reproduction, longevity and fat metabolism has been demonstrated by many investigators. A recommended daily requirement for essential fatty acids has been proposed based on a ratio of triene to tetraene fatty acids in heart tissue, erythrocytes and plasma (2).

It is now recognized that many factors, dietary and otherwise, influence the requirement for essential fatty acids. Among the dietary factors which have been shown to increase essential fatty acid requirements are certain of the non-essential fatty acids. Thus, Evans and Lepkovsky (3) reported that symptoms of essential fatty acid deficiency were aggravated when rats were fed a fat-free diet supplemented with saturated fat. These observations were confirmed using hydrogenated coconut oil in 1936 by Sinclair (4), later in our laboratory (5), by Peifer and Holman (6), and by Coleman et al. (7) using acetostearins. However, using growth and dermal symptoms as criteria, a hydrogenated triolein (iodine value 67.2) had no adverse effects on essential fatty acid deficiency (8). Although small amounts of fish oil fatty acid ethyl esters did not protect young rats from dermal symptoms, the growth rate compared favorably with that of animals receiving a similar amount of ethyl linoleate. Larger quantities of tuna, menhaden or herring oils fed to rats cured dermal symptoms and stimulated growth, indicating the presence of some essential fatty acid activity (9).

The importance of chain length of fatty acids on essential fatty acid requirement has been demonstrated by effects on rat growth. Thus myristic, palmitic or stearic acids, given for 4 weeks to rats fed a fat-free diet, had a depressing effect on growth (10). Rats fed a diet containing 20% of the medium-chain length fatty acid triglycerides (C_6 to C_{12}) grew better than animals fed a low fat diet, whereas

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[2] Present address: U. S. Department of Agriculture, Washington, D. C.

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in those fed the long-chain fatty acid triglycerides (C_{14} to C_{18}), growth was depressed. It was suggested that medium-chain length fatty acids decrease, and longer-chain fatty acids increase, the requirement for essential fatty acids (11). A similar hypothesis was also presented by Funch et al. (12, 13).

In essential fatty acid deficiency in rats, plasma cholesterol levels decrease, whereas cholesterol esters accumulate in the liver (14). In view of the evidence presented that some of the shorter-chain fatty acids might "spare" essential fatty acids, an investigation was undertaken to study the effects of fatty acid esters, from C_4 to C_{18}, on the essential fatty acid requirement, using as criteria, growth, plasma cholesterol, and hepatic cholesterol and lipid levels.

METHODS

Weanling male and female rats of our USC strain were supplied with fat-free diets supplemented as follows: group 1, no supplement; groups 2–12, supplemented orally with 100 mg/animal/day of one of the following fatty acid methyl esters, respectively: butyrate, caproate, caprylate, caprate, laurate, myristate, palmitate, stearate, oleate, elaidate, and linoleate. Details of the diet are shown in table 1.

Animals were weighed weekly and were kept on the experimental regimen for 12 weeks, at which time the females were bred with males of our stock colony and the male animals were killed, using sodium pentobarbital [4] anesthesia. Blood was removed from each animal by heart puncture and subjected to centrifugation for separation of plasma and red cells. Plasma was extracted for lipid analysis, using alcohol-acetone (1:1). Livers were excised quickly, blotted, and extracted in a Waring Blendor using the method of Thompson et al. (15). Cholesterol determinations were made on plasma and liver extracts by a modification of the Sperry-Schönheimer method (16). Total hepatic lipids were determined gravimetrically.

TABLE 1

Diet (fat-free)

	%
Sucrose	71.53
Casein	18.23
Salt mix [1]	4.0
Cellulose [2]	4.0
Vitamin mix [3]	2.0
Choline chloride	0.24

[1] Wesson modification of Osborne-Mendel formula (Science, 75: 339, 1932).

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

[3] The vitamin mixture had the following composition: (in grams) vitamin-test casein, 61.35; *p*-aminobenzoic acid, 2.42; inositol, 2.0; *α*-tocopheryl acetate, 1.3865; ascorbic acid, 0.8; thiamine, 0.288; Ca pantothenate, 0.24; niacin, 0.24; vitamin B_{12} triturate, 0.24; riboflavin, 0.11; pyridoxine, 0.108; vitamins A and D (500,000 USP/g vitamin A, 50,000 USP/g vitamin D; Crystalets, Chas. Pfizer and Co., New York), 0.052; folic acid, 0.046; menadione, 0.022; and biotin, 0.016.

DISCUSSION

Growth and mortality of male rats fed fat-free diets unsupplemented or supplemented with the various fatty acid esters are shown in table 2. Of the methyl esters tested, only animals given the oleate and linoleate supplements had a gain in weight over that exhibited by the animals given the unsupplemented diet. Supplementation with all of the other fatty acid esters from C_4 through C_{18} resulted in a weight loss when compared with the unsupplemented animals. Methyl esters of C_4, C_8 and C_{10} fatty acids appeared to have more of a depressing effect on growth than methyl esters of C_{12}, C_{14}, C_{16}, and C_{18}. The *trans* oleic acid ester, elaidate, and the C_8, caprylate, had the largest growth-depressing effect. Caprylate also caused the highest mortality. These results appear to be in contradiction to those reported by Kaunitz et al. (11) in which the mixed medium-chain fatty acid triglycerides (C_6 to C_{12}) supported better growth than was observed in animals fed the low fat diet, whereas the longer-chain length triglycerides (C_{14} to C_{18}) depressed growth. Since in Kaunitz's experiments, however, the triglycerides were fed at a dietary level of 20% and in our experiments the fatty acid esters were given as a dietary supplement at approximately a 1% level, valid comparisons cannot be made.

The effect of the various fatty acids on growth, mortality, and reproduction of female rats is also shown in table 2. In contrast with the males, there was little effect on weight when the female animals were given supplements of the various

[4] Somnopentyl, Pitman-Moore Company, Indianapolis, Indiana.

TABLE 2

Effect of fatty acid esters on growth, mortality and reproduction

Supplement	Males			Females				
	Wt gain (12 weeks)	Difference from fat-free group	Mortality	Wt gain (12 weeks)	Difference from fat-free group	Mortality	No. litters born	No. animals born [1]
	g	*g*	%	*g*	*g*	%		
1. None	193(9) [2]	—	10	138(10) [2]	—	0	0	0
2. Butyrate	162(9)	−31	10	128(9)	−10	10	0	0
3. Caproate	173(10)	−20	0	139(8)	− 9	20	0	0
4. Caprylate	140(7)	−53*	30	135(8)	− 3	20	1	6
5. Caprate	158(10)	−35	0	142(10)	+ 4	0	1	6
6. Laurate	176(8)	−17	20	132(8)	− 6	20	0	0
7. Myristate	177(10)	−16	0	133(10)	− 5	0	5	33
8. Palmitate	171(9)	−22	10	131(10)	− 7	0	6	39
9. Stearate	175(8)	−18	20	133(10)	− 5	0	5	28
10. Oleate	218(10)	+15	0	163(10)	+25*	0	5	33
11. Elaidate	138(10)	−55**	0	129(9)	− 9	10	2	12
12. Linoleate	234(10)	+41*	0	167(10)	+29*	0	10	70

[1] None of the animals survived to 3 days except those in group 12.
[2] Numbers in parentheses are numbers of animals/group.
* Significance by *t* test, $P < 0.05$.
** Significance by *t* test, $P < 0.005$.

TABLE 3

Effect of fatty acids on plasma cholesterol and hepatic cholesterol and lipid levels of male rats

	Plasma cholesterol		Hepatic cholesterol		Hepatic lipid
	Free	Total	Free	Total	
	mg/100 ml	*mg/100 ml*	*mg/g*	*mg/g*	*mg/g*
Fat-free (9) [1]	14.8 ± 1.3 [2]	45.5 ± 2.9	2.13 ± 0.05	3.76 ± 0.22	68.6 ± 3.7
+ butyrate (9)	11.7 ± 0.8	42.8 ± 2.2	2.19 ± 0.06	3.51 ± 0.24	64.3 ± 3.8
+ caproate (10)	12.9 ± 0.6	44.9 ± 1.9	2.09 ± 0.08	3.75 ± 0.24	71.4 ± 2.8
+ caprylate (7)	12.8 ± 0.4	41.8 ± 1.5	1.73 ± 0.06	3.54 ± 0.26	59.6 ± 2.5
+ caprate (10)	13.2 ± 0.3	46.0 ± 3.5	2.24 ± 0.07	4.27 ± 0.32	70.3 ± 3.8
+ laurate (8)	20.1 ± 1.3*	45.9 ± 1.4	2.50 ± 0.11***	5.85 ± 0.76***	82.5 ± 4.9*
+ myristate (10)	13.7 ± 0.9	47.6 ± 2.7	2.17 ± 0.17	3.82 ± 0.33	71.4 ± 9.2
+ palmitate (9)	11.9 ± 0.5	40.9 ± 1.7	2.04 ± 0.05	3.20 ± 0.21	55.8 ± 4.6
+ stearate (7)	13.4 ± 1.0	44.0 ± 4.0	2.08 ± 0.06	3.85 ± 0.25	65.5 ± 2.0
+ oleate (10)	16.4 ± 0.8	55.2 ± 2.2*	2.61 ± 0.11****	5.15 ± 0.50**	64.1 ± 2.6
+ elaidate (10)	12.3 ± 2.2	37.4 ± 4.0	2.12 ± 0.07	3.49 ± 0.23	54.5 ± 2.5**
+ linoleate (10)	12.8 ± 0.5	55.3 ± 1.1**	1.65 ± 0.13****	2.21 ± 0.25****	50.9 ± 1.7***

[1] Numbers in parentheses are number of animals per group.
[2] Includes SE of mean.
* Significantly different from fat-free control (by *t* test) $P < 0.05$.
** Significantly different from fat-free control (by *t* test) $P < 0.025$.
*** Significantly different from fat-free control (by *t* test) $P < 0.005$.
**** Significantly different from fat-free control (by *t* test) $P < 0.001$.

fatty acid esters, with the exception of females given oleate; in this group there was an average gain in weight of 25 g over those females fed the fat-free diet which is similar to the gain of 29 g observed in the animals receiving linoleate. However, there was a noticeable improvement in reproduction when fatty acid esters of myristate, palmitate, stearate, oleate, or linoleate were given to the females, in that the number of successful pregnancies and number of young born were much greater than when the females were fed fat-free diets either alone or supplemented with the shorter-chain fatty acid esters and elaidate. None of the young survived after 3 days except in the group given the linoleate supplement.

Although males in all groups had typical "scaly" skin conditions, those animals receiving the C_{10}, C_{14}, C_{16} and C_{18} saturated fatty acids had more severe dermal symptoms than those in the other groups. As far as the females were concerned, no skin symptoms were observed in those given the fat-free diet unsupplemented or

supplemented with C_4 and C_6 fatty acids; those groups fed the C_{12}, C_{14}, C_{16} and C_{18} saturated fatty acids developed a more pronounced dermatitis than those fed the C_8 and C_{10} saturated, and C_{18} unsaturated fatty acids.

In table 3 is shown the effect of the various fatty acid esters on plasma and liver cholesterol levels and liver total lipids. Animals fed a fat-free diet supplemented with linoleate have a significantly higher plasma cholesterol level than those fed the unsupplemented diets. Rats given oleate have a similar elevation in plasma cholesterol level but this high value is accompanied by an elevated hepatic cholesterol value as well, whereas the livers of rats given linoleate supplements are low in cholesterol content. A high hepatic cholesterol level is also observed in the animals supplemented with laurate, although this increase is not accompanied by an increased total plasma cholesterol level. Unesterified cholesterol in plasma is considerably higher in the groups given the laurate supplement than in groups fed the other fatty acid ester supplements. This may indicate an interference with cholesterol esterification in the liver. As far as the other fatty acids are concerned, none appear to have a salutary effect on either plasma or liver cholesterol values when compared with the values obtained from animals fed the fat-free diet. This is in agreement with results reported by Beveridge et al. (17) and Hashim et al. (18) who reported that saturated fatty acids containing 8- and 10-carbon atoms produce only slight effects on cholesterol concentrations of human blood. However, Grande (19) reported that saturated fatty acids of C_{12} and C_{14} induce much higher concentrations of cholesterol in the serum of dogs than do saturated fatty acids of C_8 and C_{10}. Although in the work reported here, the C_{12} acid had little effect on rat plasma total cholesterol levels, certainly its effect in elevating plasma free cholesterol levels and hepatic cholesterol content is marked. Animals given the laurate supplements also had the highest hepatic total lipid accumulation. Elaidate, which had a considerably larger depressing effect on growth, does not adversely affect cholesterol values, but does result in lower hepatic total lipid levels similar to that observed in the animals fed the linoleate supplement.

It is known that in the plasma of many species of animals, cholesterol is found preferentially esterified with linoleic acid. It has been suggested (20) that in the absence of polyunsaturated fatty acids, cholesterol is esterified with more saturated fatty acids which may not as easily enter a lipoprotein complex for subsequent transport from liver to plasma and to other tissues. However, undoubtedly other factors, such as enzyme specificity for fatty acid esterification with cholesterol, differences in rate and site of absorption of the various fatty acids under investigation, and subsequent metabolic pathways of these fatty acids of varying chain length must also be considered. An effect on cholesterol biosynthesis was eliminated since essential fatty acid deficiency results in a marked decrease in hepatic cholesterol biosynthesis (21). Further studies on fatty acid imbalances are in progress.

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Net Protein Utilization Determined in Short- and Long-term Experiments with Rats [1]

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ABSTRACT The protein quality, measured as net protein utilization, of casein (10.5 protein calories per cent total calories in the diet) and gluten (35.6 protein calories per cent total calories in the diet), was evaluated by means of short- and long-term experiments. Casein and gluten diets of equal protein value (as net dietary protein calories per cent) were chosen as representative of the amino acid limitations most frequently observed in human nutrition: sulphur-bearing amino acids and lysine. For the short-term experiment, the net protein utilization was determined by the method of Miller and Bender on 31-day-old rats. For the long-term experiment, rats were fed the diets under study from the time they were weaned until they were 133 days old. The net protein utilization was calculated on the basis of body nitrogen gain, loss of endogenous nitrogen and nitrogen intake, according to a formula proposed by the authors. The net protein utilization values for both diets determined by the short-term experiment (70.7 and 19.8, respectively, for the casein and gluten diets) are in agreement with those calculated for the long-term experiment (75.2 and 19.8). The protein value, measured as net dietary protein calories, of casein and gluten diets obtained from a 10-day experiment is in agreement with that obtained from a 112-day experiment.

Nutritionists are concerned with the determination and expression of the protein value of diets and feeds — human nutritionists because of their interest in designing new and low-cost protein foods that can be used clinically and epidemiologically in the prevention and cure of protein malnutrition, and animal nutritionists because of its economic importance to the livestock producers generally and to the poultry industry especially.

Unfortunately, most of the experimental work on the protein value of diets is difficult to evaluate and compare because of the various methods used and the different ways of expressing the results. Platt et al. (1) have proposed to express the protein value of a diet in terms of net dietary protein calories % (NDpCals %) which is the product of protein concentration by protein quality. Protein concentration (P) is expressed as protein calories per cent total calories. Protein quality is expressed as net protein utilization (NPU) and determined experimentally in a short-term (10 days) feeding trial which uses weanling rats (2).

It seemed desirable to test the long-term validity of the expression net dietary protein calories % (NDpCals %) by a long-term experiment considering that: a) the expression has been used to make dietary recommendations at different ages in the human being (1); b) that one of the factors, namely net protein utilization, is obtained in a short-term feeding experiment that uses young rats (2); and c) protein quality has been reported to change with age (3).

EXPERIMENTAL

Diets. Casein diet: (in grams) casein,[2] 130; cornstarch, 370; vegetable fat, 150; and vitamin and mineral mixture,[3] 350. The calorie value of this diet calculated from chemical analysis was 4.3 kcal/g. Casein supplied 10.5% of the total calories of the diet. Gluten diet: gluten,[4] 535; vege-

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[2] Nutritional Biochemicals Corporation, Cleveland.

[3] Vitamin and mineral mixture contained: (in kg) vitaminized carbohydrate, 1; salt mixture, 1; potato starch, 2; and glucose, 3. Vitaminized carbohydrate: (in grams) thiamine·HCl, 0.3; riboflavin, 1.0; pyridoxine·HCl, 0.2; Ca pantothenate, 6.0; nicotinic acid, 20.0; inositol, 20.0; *p*-aminobenzoic acid, 60.0; biotin, 0.02; folic acid, 0.2; vitamin B_{12}, 0.005; choline hydrochloride, 60.0; and maize starch, 5000.0. Salt mixture: (in grams) NaCl, 22; $CaHPO_4 \cdot H_2O$, 130; K citrate ($K_3C_6H_5O_7 \cdot H_2O$), 125; $MgSO_4 \cdot 7H_2O$, 30; Fe citrate, 5.0; and traces, 0.7. Traces: (in grams) KI, 12; NaF, 10; $MnSO_4 \cdot H_2O$, 2; Cu_2I_2, 1; $KAl(SO_4)_2$, 1; and $ZnSO_4 \cdot 7H_2O$, 1.

[4] See footnote 2.

table fat, 150; and vitamins and minerals [5] 350. The calorie value of this diet calculated from chemical analysis was 4.4 kcal/g. Gluten supplied 35.6% of the total calories of the diet.

The composition of the diets is shown in table 1.

Fat-soluble vitamins were given orally to the rats every other day according to the quantities used by Kodicek and Carpenter (4) in a long-term experiment.

TABLE 1

Analysis composition of diets

	Casein diet	Gluten diet
	%	%
Water	8.7	5.9
Ash	3.3	3.3
Nitrogen	1.75	6.84
Protein	11.2(N×6.38)	39.0(N×5.7)
Fat	14.8	14.9
Carbohydrate	62.0	36.9
Kilocalories/ 100 g	430	440

METHODS

The short-term determination of protein quality — as net protein utilization operative (NPUop) [6] — of the diets was carried out in duplicate according to the method of Miller and Bender (2) in a 10-day feeding trial using 31-day-old albino rats of our stock (5).

The long-term evaluation of the protein quality of the diets was performed in a 112-day feeding trial using 21-day-old male weanling rats. Each diet was given ad libitum to a group of 16 rats. The mean initial weight of the rats of both groups was 30 g. During the trial the animals were weighed once a week and the food intake was measured. After the experimental period the rats were killed and body nitrogen was determined by chemical analysis of the whole rat. To estimate the initial body nitrogen in the short- and long-term feeding experiments the carcasses of ten 31-day-old rats and nine 21-day-old rats were analyzed.

CALCULATIONS

The net protein utilization of the diet according to the method of Miller and Bender is calculated as:

$$\text{NPU} = \frac{B - B_k}{I} \qquad (1)$$

where,

B is the mean body nitrogen of rats (41-day-old), after 10 days' feeding of the experimental diet.

B_k is the mean body nitrogen of rats (41-day-old) after 10 days' feeding of a nonprotein diet.

I is the mean nitrogen intake of the animals fed the experimental diet.

This net protein utilization is also termed true net protein utilization (tNPU) as against apparent net protein utilization (aNPU) which can be calculated as:

$$\text{aNPU} = \frac{B - Bo}{I} \qquad (2)$$

where,

Bo is the mean body nitrogen of the rats at the beginning of the experimental period, B and I as in equation 1.

The difference between apparent and true net protein utilization is that the latter introduces in the calculations a necessary correction for the endogenous loss of nitrogen (En), which is estimated as the difference between the body nitrogen of the animals at the beginning of the 10-day experimental period and the body nitrogen of the rats fed a nonprotein diet after the same period, thus:

$$\text{En} = \text{Bo} - B_k \qquad (3)$$

This estimation of the endogenous nitrogen is possible and valid, though open to some theoretical criticism, for the 10-day interval that the method uses, but not for a long-term feeding trial. The reasons for this are both practical and theoretical, namely, the rats die of prolonged deprivation of protein and En is a function of body weight which varies with time and food intake in the growing animal.

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[5] See footnote 3.

[6] Platt, B. S., and D. S. Miller 1959 The net dietary protein value of mixtures of foods — its definition, determination and application. Proc. Nutrition Soc., *18:* vii.

It can be shown that independently of the length of the feeding trial

$$tNPU = \frac{R + En}{I} \qquad (4)$$

where,

R is the nitrogen retained with a diet over the period considered. But, R is equal to the difference between the body nitrogen at the end of the experimental period (B) and the initial body nitrogen (Bo),

$$\therefore R = B - Bo$$

Replacing R in equation 4 and rearranging

$$tNPU = \frac{B - Bo}{I} + \frac{En}{I} \qquad (5)$$

The first part of the second member of this equation is equal to aNPU (equation 2), and can be calculated from experimental data from short- or long-term feeding trials.

To calculate tNPU over a long period it is necessary to estimate the endogenous loss of nitrogen during the experimental period.

If $(En)i$ is the daily loss of endogenous nitrogen on day i, then we can consider that the total loss of endogenous nitrogen over a period of f days is equal to:

$$(En)f = \sum_{i=o}^{i=f} (En)i$$

Miller and Payne (6, 7) use the following expression to calculate the endogenous loss of nitrogen:

$$(En)i = M\, W_i^{0.73}$$

where,

M is the nitrogen used for maintenance, equals 250 mg/day/$W^{0.73}$ (where W is body weight in kilograms).

Wi is the body weight (in kilograms) on day i.

This last equation also can be expressed as:

$$dEn = M\, W^{0.73} dt$$

Therefore, the total loss of endogenous nitrogen over a period of f days is equal to:

$$(En)f = M \int_0^f W^{0.73} dt \qquad (6)$$

Combining equations 5 and 6

$$tNPU = \frac{B - Bo}{I} + \frac{M}{I} \int_0^f W^{0.73} dt \qquad (7)$$

We have used this formula to calculate the long-term net protein utilization, taking as:

B, the mean body nitrogen of rats (16 animals) at the end of the experiment period (21-day-old rats fed the experimental diet for 112 days).

Bo, the mean body nitrogen of 21-day-old rats (9 animals).

I, the mean nitrogen intake during the experimental period.

M, 250 mg/day/$W^{0.73}$.

O, f, time interval between 21 and 133 days.

RESULTS

Table 2 shows the body nitrogen, body weight, dietary intake, nitrogen intake and endogenous nitrogen of the rats fed the experimental diets in the short- and long-term feeding trials.

Short-term trial. The net protein utilization of the casein diet determined in 2 assays gave a mean value of 70.7 (70.5 and 70.8). The net protein utilization of the gluten diet determined in 2 assays showed a mean value of 19.8 (17.8 and 21.7) (see table 3).

Long-term trial. The long-term net protein utilization, calculated according to equation 7 for the casein diet was 75.2 and for the gluten diet was 19.8 (see table 3).

DISCUSSION

The calculation of true net protein utilization (tNPU) according to equation 7 implies the estimation of apparent net protein utilization (aNPU) and of endogenous nitrogen. Apparent net protein utilization tends to diminish with the age of the animal because the weight increase for a given interval decreases with time (or age). In other words, the apparent retention of nitrogen tends to approach zero with time. Both diets show lower aNPU for the long-term feeding trial (table 3).

Endogenous nitrogen has been shown to be a function of body weight (or area) in

TABLE 2

Age and mean weight, body nitrogen, dietary intake, nitrogen intake and endogenous nitrogen of rats fed different diets

Age	Diet	Weight	Body N	Dietary intake	N intake	Endogenous N
days		*g*	*g*	*g*	*g*	*g*
			Long-term trial			
21	Initial	30	0.77	—	—	—
133	Casein	273	7.12	1.043	18.35	7.48 [1]
133	Gluten	306	7.90	1.113	76.15	7.97 [1]
			Short-term trial			
31	Initial	64	1.68	—	—	—
41	Casein	81	2.28	70	1.23	0.37 [1]
41	Nonprotein	51	1.42	35	0.03	0.26 [2]
31	Initial	56	1.47	—	—	—
41	Gluten	76	2.14	62	4.21	0.34 [1]
41	Nonprotein	46	1.28	42	0.05	0.19 [2]

[1] Calculated according to equation 6.
[2] Calculated according to equation 3.

TABLE 3

Short- and long-term net protein utilization for casein and gluten diets

	True net protein utilization (tNPU)	Apparent net protein utilization (aNPU)
Casein ($P = 10.5$)		
Short-term	70.7	49.0
Long-term	75.2	34.8
Gluten ($P = 35.6$)		
Short-term	19.8	16.1
Long-term	19.8	9.4

various species (8). The validity of this function conditions the validity of the estimation of endogenous nitrogen and therefore of long-term tNPU according to equation 7. Miller and Payne (6), working with rats, use 250 mg N/day/$kg^{0.73}$ when calculating endogenous nitrogen from body weight. We have calculated this constant from the loss of body nitrogen in rats fed the nonprotein diet in 14 routine NPU assays and found it to be 199 mg N/day/$kg^{0.73}$, which can be considered of the same order as the previous figure. The agreement between the calculated long-term and the short-term tNPU appears good using either of the constants: 75.2 and 67.8 vs. 70.7 for casein; 19.8 and 17.8 vs. 19.8 for gluten.

We have attempted to show the more general validity of equation 7 applying it to the data of our short-term feeding trial. The performance of the animals estimated in this way is comparable with that of the rats on the long-term feeding experiment, namely, 79.0 vs. 75.2 for casein and 24.0 vs. 19.8 for gluten, using $M = 250$ mg N/day/$kg^{0.73}$; 73.0 vs. 67.8 for casein and 22.3 vs. 17.8 for gluten, using $M = 199$ mg N/day/$kg^{0.73}$.

The estimation of tNPU according to equation 1 uses as measure of endogenous nitrogen the loss of body nitrogen of a group of rats fed a nonprotein diet. This would tend to underestimate endogenous nitrogen (and therefore tNPU) in the growing animal, and somewhat higher values should be expected (and were found) for tNPU when applying equation 7 to the calculation of the short-term data.

Miller and Bender (2) introduce a correction for the nitrogen intake of animals fed the nonprotein diet when calculating tNPU:

$$\text{tNPU} = \frac{B - B_k + I_k}{I}$$

where B, B_k and I are as in equation 1 and I_k is the nitrogen intake of the rats fed the nonprotein diet. If this correction is applied, the agreement between the corrected short-term and long-term value is even better for casein (70.7 to 72.3 vs. 75.2) and slightly worse for gluten (19.8 to 21.7 vs. 19.8) than when the correction is not applied.

Diets of equal protein value (as NDp-Cals %) containing casein and gluten were

chosen. These proteins represent the most frequent types of amino acid limitation observed in human and animal diets: sulphur-bearing amino acids (casein) and lysine (gluten).

There has been much discussion as to whether the essential amino acid requirements vary during different stages of life. If the requirement of a certain essential amino acid varies with age, for a diet whose protein is limited by that amino acid, different protein values would be found depending on the age of the experimental animal.

As to the requirements of sulphur-bearing amino acids, it is reported in the literature that: a) young and adult animals have the same requirements (9); b) adult animals have higher requirements than young ones (10, 11); and c) young animals have higher requirements than adult ones (12). Thus, Forbes and Yohe (9) observed that the requirements of sulphur-bearing amino acids, expressed as percentage of total nitrogen ingested, are the same for young and adult rats; Mitchell and Beadles (10) and Hartsook and Mitchell (11) reported a higher requirement of sulphur-bearing amino acids, expressed as percentage of the protein intake, for adult rats than for young ones; Henry and Kon (12) suggest that the requirements of sulphur-bearing amino acids decrease with the age of the rat.

However, according to some reports (13), the relative requirements of sulphur-bearing amino acids, expressed in relation to tryptophan, are higher for adult men than for children.

Reports in the literature are conflicting. FAO (13), attempting to simplify a complex problem, calculate the requirements in terms of a type of protein, namely, they keep constant the relative proportions of essential amino acids, varying only the quantity of protein for each age.

Another point of controversy is that of the requirements of lysine; some authors are of the opinion that they are similar for the adult and for the young, whereas others state that they are higher in the young.

Neuberger and Webster (14) showed that the need for lysine is not as great in adult rats as in growing ones. Henry and Kon (3) experimented with bread proteins on young and adult rats and they observed that lysine deficiency is relatively higher in the young animal. Further experiments by the same authors (12) appear to confirm that lysine requirement is higher for the young than for the adult animal. Forbes and Yohe (9), Mitchell and Beadles (10) and Jansen (15) agree with the other authors cited.

However, the lysine need expressed in relation to tryptophan is quite similar in adults and in children (13). Furthermore, Snydermann and Holt,[7] working with humans, observed no experimental support for the requirement of higher proportion of lysine during growth period.

The short-term determination of protein quality is in agreement with the results of the long-term feeding trial, namely, 70.7 vs. 75.2 for casein and 19.8 vs. 19.8 for gluten. These values may be considered comparable, since in ten previous NPU assays the same diet gave the following values: average, 71.3; mean error $\pm$ 0.8, and standard deviation $\pm$ 2.6. Comparable results could be interpreted as meaning that the requirements for sulphur-bearing amino acids and lysine in rats are essentially the same through a considerable part of their life.

The initial age of the rats on the short- and long-term experiments was different (table 2). The long-term experiment covers the period from 21 to 133 days of life, that is, 10 days before and 92 days after the short-term feeding trial (31 to 41 days of age). The long-term experiment covers periods of life with possible different requirements (3, 9–15) making the agreement found all the more valid.

The results shown support the theory that the protein value of a diet remains constant throughout a considerable part of the life of the rat but may not have a general validity for other proteins or other protein concentrations.

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Myocardium and Plasma Electrolytes in Dietary Magnesium and Potassium Deficiency in the Rat [1]

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ABSTRACT Deficiencies in the diet of magnesium and of potassium were studied in young growing rats. The metabolism of magnesium and potassium are interrelated, and factors affecting this interrelationship are reviewed. A deficiency of magnesium in the diet produced a decrease in cardiac muscle potassium and plasma potassium, a decrease in plasma magnesium (two of three experiments), and a decrease in muscle magnesium (one of two experiments). Dietary potassium deficiency produced an unexpected increase in plasma magnesium, a decrease in cardiac muscle magnesium and a decrease in cardiac muscle and plasma potassium. From our experiments and from the work of others we conclude that a dietary deficiency of either magnesium or potassium produces a decrease in the concentration of these ions in cardiac muscle and in plasma. The one exception is that of an increase in plasma magnesium with dietary potassium deficiency. The hypothesis that these interrelationships are related to reactions involving energy production and ultilization is considered likely.

Most cells are characterized by their ability to maintain a high intracellular concentration of potassium in the presence of a lower concentration of this ion in extracellular fluid. As the principal cation in intracellular water, potassium plays an important role in the maintenance of osmotic pressure and acid base balance. The high intracellular potassium level is maintained, presumably, by energy processes within the cell or cell membrane. In animals rendered potassium-deficient, histopathologic changes of the kidney and heart result, and in the case of the latter organ, the normal electrical conductivity is apparently associated with an unequal distribution of potassium between the intracellular and extracellular spaces (1, 2).

Magnesium is also present in relatively high concentrations within the cell and it is exceeded in amount only by potassium. Cotlove et al. (3) were the first to demonstrate that rats made deficient in magnesium by dietary means developed a secondary deficiency of potassium within the cell. This observation has been confirmed by MacIntyre and Davidsson (4), Manitius and Epstein (5), and Whang and Welt (6) but not by Smith et al. (7). Most investigators have suggested that the decrease in intracellular potassium in magnesium deficiency was due to a defect in ion transport brought about by an impairment in the energy yielding reactions (4, 6, 8, 9).

Vitale et al. (8) and Seta et al.[2] reported an uncoupling of oxidative phosphorylation in mitochondria isolated from the heart of magnesium-deficient rats and Baltscheffsky (10) showed that liver mitochondria bathed in a magnesium-free reaction medium swelled with uncoupling of oxidative phosphorylation. Nakamura et al. (11) showed, by electron microscopy, morphological changes and swelling of liver mitochondria isolated from magnesium-deficient rats.

The effect of potassium deficiency on magnesium metabolism has not been studied as extensively as the effect of magnesium deficiency on potassium metabolism.

We decided to continue previous studies on the interrelationships of dietary potassium and magnesium deficiency because

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certain results in the literature were in conflict with other results, or appeared unlikely. For example, in dietary magnesium deficiency, plasma potassium has been reported to be unchanged. That intracellular magnesium remains unchanged or increased in dietary potassium deficiency appeared to require confirmation. We also thought that a clinically more meaningful tissue to study would be cardiac rather than skeletal striated muscle.

The present studies deal with dietary deficiencies of potassium or magnesium, or both, in young growing rats.

EXPERIMENTAL PROCEDURE

Three separate experiments were carried out at different times. In all 3 experiments, male albino CD rats obtained from the Charles River Breeding Laboratories were used. All animals were housed in individual cages with screen bottoms and were fed and watered ad libitum.

Experiment 1. Rats weighing 70 g were fed a commercial diet for a period of 5 days. At the end of this time they were divided into 4 groups of 8, averaging 100 g/rat. They were fed a purified diet which consisted of the following ingredients per 100 g: casein (vitamin-free), 20.0; glucose, 62.5; corn oil,[3] 9.0; cod liver oil, 1.0; cellulose,[4] 3.0; salt mix, 2.2; vitamin mix, 0.5; $CaCO_3$, 0.5; choline chloride, 0.3; and cholesterol, 1.0. The vitamin mix using glucose as a carrier, provided the following mg/kg of diet: thiamine·HCl, 4; riboflavin, 8; pyridoxine·HCl, 4; niacinamide, 40; Ca pantothenate, 25; folic acid, 1; biotin, 0.2 and menadione, 01.

Table 1 illustrates the design of the experiment, the number of animals per group, and the level of dietary Mg and K fed each group. In this and the subsequent experiments, magnesium was added to the diet in the form of MgO and potassium as K_2CO_3. The basal diet prior to the addition of K and Mg contained less than 2 mg of Mg and less than 10 mg of K per 100 g. The concentrations of other minerals fed in this experiment were the same as those provided by diets containing 4% of the Jones and Foster (12) salt mixture. The diet contained the following in

[3] Mazola, Corn Products Company, New York.
[4] Cellu Flour, Chicago Dietetic Supply House, Chicago.

TABLE 1

Effect of Mg and K deficiency on weight and tissue and plasma electrolyte levels

	Dietary		Wt gain	Myocardium				Plasma			
	Mg	K		Mg	K	Na	Cl	Mg	K	Na	Cl
	mg/100 g		*g*	*mEq/kg wet tissue*				*mEq/liter*			
Exp. 1											
	6	100	82 [1]	10.9	71.0	52	4.5	0.60	1.8	146	103
	6	800	94	15.5	74.9	47	4.2	0.40	3.8	147	105
	96	100	119	11.5	74.3	45	4.6	3.02	2.8	148	102
	96	800	160	15.7	84.4	41	3.8	2.25	4.8	149	103
Exp. 2											
	6	50	38 [1]	7.97	72.7	50	3.5	0.61	3.3	146	91
	6	100	44 [1]	8.60	78.3	47	3.3	0.83	3.6	146	96
	6	500	41 [1]	8.87	81.3	42	3.3	0.49	4.3	145	104
	6	800	61 [1]	8.77	81.3	39	3.4	0.43	4.7	147	100
	12	50	41 [1]	8.07	76.3	51	3.5	0.90	2.9	143	94
	12	100	49	7.80	82.0	51	3.7	0.60	3.6	148	99
	12	500	88	9.33	86.3	40	3.5	0.83	4.7	146	103
	12	800	74	9.10	87.3	39	3.7	0.85	4.6	145	104
	48	50	41 [1]	7.20	78.3	46	3.7	1.82	3.9	146	91
	48	100	71	7.93	83.7	46	3.8	1.72	3.2	147	98
	48	500	92	9.77	84.7	37	3.7	1.35	4.2	146	105
	48	800	82	9.07	86.7	40	3.8	1.35	4.9	144	104
	96	50	46 [1]	7.50	78.0	46	3.5	1.79	3.4	145	92
	96	100	82	10.73	82.7	43	3.6	1.79	3.9	146	97
	96	500	117	10.63	86.7	40	3.6	1.58	5.5	144	100
	96	800	112	10.93	87.3	39	3.6	1.30	5.4	145	104

[1] Ten animals per group; all others contained 8 rats per group. Experimental period was 19 to 20 days.

mEq/kg: Na, 90; Ca, 320; Cl, 90; and P, 106. After 19 to 20 days, the rats were anesthetized with ether and through an abdominal incision blood was drawn from the abdominal aorta with a heparinized syringe. The following plasma constituents were determined: magnesium (13), calcium (14), sodium and potassium by flame photometry,[5] and chloride (15).

The kidney and the apex of the heart were fixed in 10% formalin and examined microscopically. Stains included hematoxylin and eosin and periodic acid Schiff. Occasional slides were stained with von Kossa, Alizarin red S, and PAS diastase. The left kidney of each animal was weighed. The remainder of the heart was cut transversely and each part was weighed. One of these was homogenized in 10 volumes of water, and the other portion was dried to constant weight in an oven set at 90°. The homogenate was allowed to stand 72 hours at 5°, centrifuged, and the supernatant was analyzed for Na, K, Mg, and Cl by the methods described above. The results are expressed as milliequivalents per kilogram of wet tissue. The efficiency of the method used for extraction of the tissue was compared with a digestion method and an ash procedure. The 3 procedures gave comparable results, and because of the simplicity of the first method, this one was used. The above method for analysis of tissue electrolytes and tissue water was also carried out in the subsequent experiment.

Experiment 2. In this experiment rats weighing approximately to 50 g were fed commercial laboratory chow[6] for several days after which time they were divided into 16 groups, averaging 75 g/rat. They were fed the basal ration listed above to which were added varying amounts of Mg and K. These animals were killed after 21 days.

Experiment 3. In this experiment weanling rats were fed commercial laboratory chow[7] for 7 days, were divided into 4 groups averaging from 77 to 79 g/rat, and were fed, ad libitum, the basal diet (exp. 1) containing levels of Mg and K as indicated in table 2. There were 30 animals per group; 10 animals from each group were killed as described above after 1, 2 and 3 weeks. Cardiac tissue was not analyzed but rather a portion of the ventricle,

[5] Baird-Atomic, Inc., Cambridge, Massachusetts.

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

[7] See footnote 6.

TABLE 2

Effect of dietary K and Mg on electrolytes and morphologic changes

Dietary Mg, mg/100 g Dietary K, mg/100 g	6 50	6 500	96 50	96 500
After one week:				
1. Weight gain, g	14.9	27.3	16.1	32.0
2. Serum K, mEq/liter	2.7	4.4	2.6	4.6
3. Serum mg, mEq/liter	1.13	0.87	2.28	1.92
4. Left kidney × 100/body wt	0.632	0.440	0.643	0.452
5. Renal K deficiency	10/10	0/10	10/10	0/10
6. Heart lesions	1/10	3/10	1/10	0/10
After 2 weeks:				
1. Weight gain, g	22.1	59.4	30.1	77.2
2. Serum K, mEq/liter	1.95	3.7	2.3	4.3
3. Serum Mg, mEq/liter	1.93	0.62	2.23	1.67
4. Left kidney × 100/body wt	0.707	0.440	0.628	0.429
5. Renal K deficiency	9/9	0/10	10/10	0/10
6. Heart lesions	3/9	2/10	2/10	1/10
After 3 weeks				
1. Weight gain, g	30.1	82.5	50.7	120.6
2. Serum K, mEq/liter	2.4	4.2	2.2	4.4
3. Serum Mg, mEq/liter	0.97	0.62	2.28	1.68
4. Left kidney × 100/body wt	0.745	0.456	0.649	0.395
5. Renal K deficiency	9/10	0/10	10/10	0/10
6. Heart lesions	9/10	2/10	4/10	0/10

other than the apex, was used for microscopic examination.

The results of all experiments were analyzed for statistical significance by covariance analyses (16).

RESULTS

Growth. Tables 1 and 2 show the weight gain of the animals in the various groups from experiments 1, 2 and 3. In experiments 1 and 3, animals fed diets deficient in either Mg or K grew relatively less than those animals fed the diet containing the highest level of Mg and K. In experiment 2, at any level of dietary Mg an increase in the level of dietary K above 50 mg/100 g resulted in an increased weight gain. The difference in weight gain between the groups fed at the two highest levels of K lacked statistical significance. Thus, under the conditions of experiment 2, the K requirement appeared to be somewhere between 100 and 500 mg/100 g of diet.

Nevertheless, at any level of dietary K, maximal weight gain did not appear to be obtained at the highest level of Mg fed. At every level of dietary K, an increase in the level of dietary Mg from 48 to 96 mg/100 g resulted in a significant gain in weight ($P < 0.05$). Therefore, the dietary Mg requirement for maximal weight gain in this experiment (exp. 2) may not have been obtained.

Plasma electrolytes. Table 1 lists the concentrations of various electrolytes observed in the plasma and in heart tissue.

The effect on plasma electrolytes in experiment 1 can be summarized as follows: low dietary Mg produced hypomagnesemia and low dietary K produced hypokalemia as expected. In addition, and of greater moment, the low dietary Mg produced hypokalemia and low dietary K produced a rise in serum Mg. All of these changes were statistically significant ($P < 0.05$).

The plasma Mg decreased from 3.02 to 0.60 mEq/liter with a decrease in dietary Mg from 96 to 6 mg/100 g, the dietary K being 100 mg/100 g. A similar decrease was noted with a decrease in dietary Mg with the dietary K being 800 mg/100 g.

The plasma K decreased from 3.8 to 1.8 mEq/liter with a decrease in the dietary K from 800 to 100 mg/100 g the dietary Mg being 6 mg/100 g. A similar decrease was noted with decrease in dietary K with the dietary Mg being 96 mg/100 g.

The plasma K decreased from 2.8 to 1.8 mEq/liter with a decrease in the dietary Mg from 96 to 6 mg/100 g, the dietary K being 100 mg/100 g. Similarly, the plasma K decreased from 4.8 to 3.8 mEq/liter with a decrease in the dietary Mg from 96 to 6 mg/100 g, the dietary K being 800 mg/100 g.

The plasma Mg increased from 2.25 to 3.02 mEq/liter with a decrease in the dietary K from 800 to 100 mg/100 g the dietary Mg being 96 mg/100 g. The similar phenomenon at the lower level of dietary Mg lacked statistical significance.

There were no statistical differences in the concentrations of serum Cl or serum Na between groups in experiment 1.

Table 1 shows the changes in serum electrolytes in the rats of experiment 2. Covariance analysis (table 3) of the data obtained on serum electrolytes allowed the following conclusions: dietary deficiency of Mg produced hypomagnesemia and dietary deficiency of K produced hypokalemia. In addition, dietary Mg deficiency produced hypokalemia. Dietary K deficiency tended to produce an increase in serum Mg although the effect was of borderline statistical significance ($0.10 > P > 0.05$). No significant interaction was noted; i.e., no synergistic effects between dietary K and Mg on serum K were observed.

The plasma Cl level appears to be a function of dietary K and not of dietary Mg. Regardless of the level of dietary Mg, increase in the K content of the diet from 50 to 100 to 500 or 800 mg/100 g resulted in an increase in plasma Cl concentration. With respect to serum Na, no differences were noted in concentration between groups.

Table 2 illustrates the changes in serum electrolytes in the rats of experiment 3 after 1, 2 and 3 weeks. The results can be summarized as follows: low dietary Mg produced hypomagnesemia and low dietary K produced hypokalemia. Low dietary Mg had no effect upon plasma K. However, low dietary K produced an increase in plasma Mg ($P < 0.01$). These effects were apparent even at one week.

Cardiac electrolytes. In experiment 1 a decrease in dietary Mg at either level of

dietary K produced no change in content of cardiac Mg. A decrease in dietary K produced a significant decrease in heart K. Additionally, a decrease in dietary Mg produced a significant decrease in heart K and conversely a decrease in dietary K produced a decrease in heart Mg.

Covariance analysis of cardiac electrolyte data of experiment 2 is provided (table 4). The dietary Mg deficiency lowered not only the concentration of Mg in heart tissue but also that of K. Dietary K deficiency similarly lowered not only the concentration of K in heart tissue but also that of Mg. No interaction of dietary K and Mg on heart Mg was noted. However, there was a synergistic effect of dietary K and Mg on heart muscle K.

The average water content of cardiac tissue in all animals from experiment 1 and experiment 2, regardless of the diet fed, was 77.6% with a range from 76.1 to 78.7%. Dietary K deficiency resulted in increased levels of Na in the heart tissue. Similarly, dietary Mg deficiency also caused an increase in heart Na but this effect of Mg deficiency was limited to the animals fed at the two lowest levels of dietary K. The increase in the Na content of the heart undoubtedly reflects the changes in the intracellular compartment inasmuch as the Cl level observed for heart tissue was low and equal for all groups. Upon subjecting all of the electrolyte data to the formulae used to calculate intracellular concentrations it becomes readily apparent that the

TABLE 3

Statistical analyses (exp. 2)

	df	Sum of mean square	Mean square	F	P value
Effect of dietary K and Mg on serum Mg					
Dietary K, mg/100 g	3	0.9301	0.3	2.77	> 0.05
Dietary Mg, mg/100 g	3	14.5106	4.6	41.1	< 0.01
Between groups		16.3573			
Within groups	32	3.5839	0.1119		
Interaction	9	0.9166	0.1017	0.908	> 0.05
Effect of dietary K and Mg on serum K					
Dietary K, mg/100 g	3	5.6542	1.8852	9.821	< 0.01
Dietary Mg, mg/100 g	3	24.7437	8.2479	42.93	< 0.01
Between groups		32.6692			
Within groups	32	6.15	0.1921		
Interaction	9	2.27	0.252	1.3	> 0.05

TABLE 4

Statistical analyses (exp. 2)

	df	Sum of mean square	Mean square	F	P value
Effect of dietary K and Mg on heart muscle Mg					
Dietary K, mg/100 g	3	633.98	211.33	25.33	< 0.01
Dietary Mg, mg/100 g	3	220.10	73.36	8.79	< 0.01
Between groups		877.00			
Within groups	32	267.01	8.34		
Interaction	9	22.97	2.56	0.31	> 0.05
Effect of dietary K and Mg on heart muscle K					
Dietary K, mg/100 g	3	28.58	9.53	14.46	< 0.01
Dietary Mg, mg/100 g	3	17.97	5.99	9.09	< 0.01
Between groups		61.53			
Within groups	32	21.00	0.66		
Interaction	9	14.98	1.67	2.53	< 0.05

changes do represent those in the intracellular space. There were no statistical differences between Cl concentrations of heart tissue in any of the groups in experiments 1 and 2. The levels observed for intracellular K are quite similar in experiments 1 and 2, although the intracellular concentrations of Mg appear to be higher in experiment 1.

Morphological observations (table 2). Dietary K deficiency produced a relative (and absolute) increase in renal weight. The results are similar to the unpresented data obtained in experiments 1 and 2. The kidney sections were evaluated as unknowns for renal signs of K deficiency by use of the following criteria: medullary tubular epithelial changes including the presence of intercalated cells, cells with abundant cytoplasm irregularly protruding into the lumen, and apparent increase in pyknosis and mitosis; PAS-positive granules in tubular cells and intertubular cells of the outer medulla; and PAS-positive granules in collecting tubules, interstitium and pelvic epithelium of the inner medulla. Virtually all of the rats fed K-deficient diets showed these renal lesions of K deficiency after 1, 2 or 3 weeks, whereas none of the rats with higher dietary K, 500 mg/100 g, showed such lesions.

Dietary Mg deficiency produced after 2 weeks a small increase in renal weight relative to body weight. Intraluminal calcium deposition in the outer medulla and deep inner cortex was absent in rats fed diets containing 96 mg Mg/100 g. Such calcium deposition was noted in rats fed a Mg-deficient diet, and at a high level of dietary K, as compared with a low level of dietary K, appeared to increase the frequency (9/10 vs. 3/10) and magnitude thereof. The calcium deposition was, at most, only minimal. Furthermore, in experiment 2, less than 10% of the Mg-deficient rats showed calcium deposition; from these and other experiments we have concluded that renal calcium deposition in Mg deficiency depends in part on factors other than Mg.

The cardiac lesions were patchy and showed varying combinations of myocytolysis, edema, neutrophilic response and particularly an increased number of mononuclear cells, some of which with indistinct cytoplasm had nuclei indistinguishable from those of nearby myocardial cells. No distinguishing difference could be determined between the cardiac lesions of rats deficient in K or Mg. It is apparent that the combined deficiency after 3 weeks produced a greater number of such lesions than did any other diet.

Other observations. Hyperemia of the ears, a well-known sign of Mg deficiency, was looked for and recorded in experiment 2 and found in those animals fed at the two lowest levels of dietary Mg. However, this sign had its onset earliest (7 to 10 vs. 18 to 20 days) and occurred more frequently (100 vs. 55%) in the 2 groups fed at the highest level of K as compared with those fed at the lowest levels of this cation. None of the rats in any of the groups developed diarrhea while supplied with the experimental diets.

DISCUSSION

Several results of the studies presented are in agreement with those published by other investigators. Mg deficiency has been shown to result in a decreased concentra- of K within the intracellular compartment despite the adequacy of the diet in K (3–6). There is good evidence that this deficit of intracellular K is not the result of kidney dysfunction (5, 6), but rather is probably due to a disruption of the mechanisms involved in maintaining the concentration gradient between intracellular and extracellular K levels (4, 6).

The relationship between oxidative phosphorylation and ion transport appears to be fairly well established (17–24). Magnesium is essential for oxidative phosphorylation, and an uncoupling of this reaction occurs in heart mitochondria isolated from Mg-deficient rats (8). Similarly, Whang and Welt (6) demonstrated that intact rat diaphragms when bathed in a K-free media lost K and that this loss could be significantly reduced by the addition of Mg to the media. Even in the presence of K in the media there was a significant loss of tissue K when Mg ions were omitted from the bath. Other studies have been carried out in other species of animals (dog and monkey) (9, 25), and the results have indicated that Mg is necessary for the maintenance of K within the cell. In these studies,

dogs and monkeys made hypomagnesemic developed marked sensitivity to administered cardiac glycosides.

Skou (21), and Post et al. (26), Dunham and Glynn (27) and Glynn (18) showed that membrane adenosine triphosphatase (ATPase) activity is intimately linked to transport of ions and that ATPase activity of nerve cells and red blood cell membranes is Mg-dependent. Their results strongly support the view that Mg deficiency results in a loss of intracellular K.

Dietary K deficiency resulted in decreased tissue and extracellular K concentrations but there is little reported in the literature concerning the mechanisms involved. If an essential nutrient is not fed, it would be expected that decreased concentrations of the nutrient would occur within the body. However, if Mg deficiency disrupts ATPase activity of cell membranes causing a loss of intracellular K, is it not equally valid to suppose that K deficiency or exclusion of K from the media also decreases ATPase activity of cell membranes causing a loss of intracellular K as well as Mg? Dunham and Glynn (27, 18) have reported that ATPase activity of red blood cell membranes is diminished not only in the absence of Mg but in the absence of K as well. In fact, in the studies reported by Whang and Welt (6), although these investigators failed to mention it, the addition of K to the media resulted in a significant retention of diaphragm Mg although no Mg was added to the media. Conversely, in the absence of Mg from the bath, there was a greater loss of tissue Mg when K was omitted. However, in the in vivo studies of Whang and Welt (6), rats fed vitamin K-deficient diets showed a decrease in muscle magnesium as calculated from their data.

Cotlove et al. (3), however, reported no change in the magnesium content of skeletal muscle in potassium-deficient animals. We, nevertheless, observed a significant decrease in cardiac Mg in rats fed low potassium diets.

In experiment 1 of the present studies, Mg deficiency was not associated with a decrease in cardiac Mg, whereas a decrease in tissue Mg was observed in experiment 2. Although tenuous, the explanation for absence of a decrease in tissue Mg in experiment 1 may be related to the fact that these animals were larger initially and may have had greater Mg stores to begin with. Perhaps for these and other reasons not readily apparent, the feeding of a diet containing a high level of dietary potassium prevented such a decrease in tissue Mg in animals fed a Mg-deficient diet.

The effect of dietary Mg deficiency on muscle Mg is not clear; three out of four studies by other investigators demonstrated a decrease in muscle Mg, and the fourth showed a decrease in tissue Mg in rats fed

TABLE 5

Observed effect of dietary deficiencies of Mg and K on muscle and plasma levels

Authors	Dietary Mg deficiency				Dietary K deficiency			
	Muscle [1]		Plasma		Muscle [1]		Plasma	
	Mg	K	Mg	K	Mg	K	Mg	K
Cotlove et al. (3) (1951)	↓	↓	NT [2]	—	—	↓	NT	↓
MacIntyre and Davidsson (4) (1958)	↓	↓	↓	—	END [2]	END	END	END
Smith et al. (7) (1962)	↓	—	↓	—	END	END	END	END
Manitius and Epstein (5) (1963)	NT	↓	↓	—	NT	↓	↑	↓
Whang and Welt (6) (1963)	↓ [3]	↓	↓	—	↑	↓	—	↓
Seta et al. (present studies)	—	↓	↓	↓	↓	↓	↑	↓
	↓	↓	↓	↓	↓	↓	—	↓
	NT	NT	↓	—	NT	NT	↑	↓

[1] Present studies used cardiac muscle; all other studies used skeletal muscle.
[2] NT = not tested; END = experiment not done; ↑ = increase; ↓ = decrease; — = no change ($P > 0.05$).
[3] Three out of four experiments.

Mg-deficient diets in three out of four experiments (table 5). That K deficiency produces hypokalemia and that Mg deficiency resulted in hypomagnesemia is well-known.

An unexpected result was observed in experiment 1 and 3. In these experiments there was a significant increase in serum Mg in rats fed K-deficient diets. In experiment 2, this increase in serum Mg lacked statistical significance ($P > 0.05$). We submitted the data of Manitius and Epstein (5) to statistical procedures and concluded that dietary K deficiency produced a significant increase in serum Mg; this was not indicated by the authors. A similar statistical procedure was carried out with the data of Whang and Welt (6) and the increase in serum Mg was not significant.

Whether the observed increase in plasma magnesium in potassium-deficient animals is related to renal function is yet to be determined.

Finally, the effect of Mg deficiency on plasma K was not consistent in our experiments. There was a significant decrease in plasma K as a result of Mg deficiency in two out of three experiments. Other investigators have shown no change in plasma K in dietary Mg deficiency. It is highly unlikely that our results can be attributed to a deficient potassium intake resulting from low food intake inasmuch as (a) this effect occurred in animals whose dietary potassium concentration was two to four times that considered minimal (28), and (b) it is a general truism that a caloric deficiency tends to "protect against" rather than to elicit manifestations of a deficiency of other constituents.

The data of our experiments strongly suggest that intracellular and extracellular Mg and K levels are all influenced by dietary Mg and dietary K deficiency. The studies of others suggest an interrelationship but none have shown as complete an interrelationship of these 2 ions on plasma and muscle levels as indicated in the present studies.

Although there are numerous references to a sodium-K pump, very little mention, or none, has ever been made of a Mg pump even though the presence of a high intra- to extracellular Mg concentration would heavily prejudice the prediction of the existence of such a pump. Indeed, it might be speculated that there is one pump for at least Na, K and Mg.

We are well aware that the interrelationship between K and Mg metabolism is not and cannot be simply a function of the concentration of only these 2 dietary variables. Just as the plasma and tissue values of one ion have been shown to be a function of other ions, and in fact, other variables, so too are the morphologic lesions of ion deficiencies dependent upon a variety of variables (4, 29); these include absolute and relative amounts of various dietary ions (30). Thus, rats deficient in K showed more striking renal hyperplasia and tubular changes if the diet was, in addition, NaCl-deficient (31). Craig and Schwartz (32) reported that K-deficient rats given Na as NaCl developed cardiac necrosis, whereas similarly treated rats given Na as $NaHCO_3$ developed no cardiac necrosis.

Weight gain or, conversely, tissue catabolism also affects the morphologic and biochemical manifestations of dietary deficiencies. Thus, to obtain a positive nitrogen balance and consistent results Spargo (33) and Spargo et al. (34) first depleted 3-month-old rats to 75% of their original weight and then tube-fed a synthetic diet containing amino acids as the source of nitrogen. In the present experiment, the rats were young and fed ad libitum. The renal lesions of K deficiency developed fairly rapidly but the cardial lesions lagged (exp. 3). Nevertheless, in view of the extensive work of Selye (35) on the cardiotoxic effects of combinations of various hormones, phosphate, K, Mg, and various stresses, we have some reservations about using depleted animals.

It should be emphasized that our K-deficien diets are only moderately deficient compared with those of most investigators. Our low K diets provided 50 mg/100 g of diet, whereas most investigators use levels of 1 to 10 mg/100 g. Kornberg and Endicott (28) concluded that a diet containing 170 mg of K/100 g was the minimal requirement for optimal growth and prevention of lesions. Conceivably, the fact that the K deficiency in the present experiment was a moderate one may be related to the eliciting of the observed K-Mg interrelationships.

The relationship of dietary Mg deficiency to calcium metabolism is a moot one. MacIntyre and Davidsson (4) reported that rats fed Mg-deficient diets developed a hypercalcemia, and further that nephrocalcinosis occurred only in the presence of hypercalcemia. Our present and previous experience is contrary to both parts of their observations (36). Thus, in the present experiment Mg deficiency did not produce hypercalcemia. The plasma Ca, 9 to 10 mg/100 ml, was relatively constant in all groups; nevertheless, the Mg-deficient rats did show a modest (morphologic) nephrocalcinosis. Forbes (29) also observed no hypercalcemia in Mg deficiency and in fact further found that comparison of the Ca, Mg and P content and pH of the urine did not reveal a basis for prediction of renal calcification; his results on the effect of Mg deficiency upon urinary Ca and P were in striking contrast with those of Smith et al. (7).

This cursory survey of the relation of some other factors to the metabolism of K and Mg indicates that studies by us and others all have, of necessity, taken a varyingly limited view of a complex problem. Future studies on the effect of dietary deficiencies of these ions upon tissue levels should also include evaluation of the energy yielding systems.

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Iron and Copper Utilization in Rabbits as Affected by Diet and Germfree Status [1]

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ABSTRACT Germfree rabbits maintained with steam-sterilized diets containing moderate amounts of iron and copper from natural ingredients but relatively high amounts of these minerals as supplemental salts, showed low hemoglobin, hematocrit and plasma iron levels, and high plasma total iron-binding capacity (TIBC) values. Plasma copper values were normal. Either conventionalization of these germfree rabbits or transfer to a steam-sterilized diet which contained more iron from natural ingredients (soybean meal) but less total amount of this mineral alleviated all symptoms of iron deficiency anemia within 4 weeks. The effect of feeding this last diet to germfree and conventional rabbits from weaning was then investigated. After the diet had been fed for 8 weeks, plasma copper, iron, TIBC, and percentage saturation of iron-binding protein levels were similar in germfree and conventional rabbits. Hemoglobin and hematocrit values were slightly lower but within normal range in germfree rabbits. The iron content of liver, spleen and kidney was lower in germfree rabbits than in conventional rabbits. There was no difference between the copper content in these organs. It is concluded that germfree rabbits utilized iron more efficiently from a natural source than from the usual mineral suuplement.

Germfree rabbits showed poor post-weaning growth and no reproduction when fed the modified commercial-type diets previously reported (1). When a successful germfree rat and mouse diet L-462 (2) was fed to germfree rabbits, it was found necessary to increase the cations, especially for magnesium and potassium, in order to prevent severe aciduria.[3] Increasing the water content of this formula L-473E5 to about 30% prior to autoclaving improved vitamin retention (3) and permitted the first successful reproduction by germfree rabbits.

In an effort to reduce the browning of this diet during sterilization, the skim milk powder was omitted, and the levels of iron and copper were reduced from what appeared to be unnecessarily high levels. The resulting diet L-477 showed much less tendency to brown. It supported growth and reproduction of germfree and conventional guinea pigs (4) and of conventional rabbits. Germfree rabbits fed this diet, however, showed bone fragility, severe anemia and irregular pregnancies, and were unsuccessful in nursing their young. The anemia was of the microcytic and hypochromic type which in rabbits has been associated with a deficiency of iron, copper or both (5).

Hove and Herndon (6) observed that rabbits have high requirements for potassium and they associated bone fragility with a deficient potassium intake. Thacker (7) demonstrated that, in rabbits, a basal ration which produced neither normal hemoglobin nor adequate bone ash levels was corrected when supplemented with salts of sodium, potassium, calcium or magnesium carrying an anion metabolized to CO_2 and H_2O. Added as chlorides or sulfates, this cation supplementation showed no effect. Comparing casein and soybean meal diets for rabbits, Hove and Herndon (8) had observed that defatted soybean meal (commercial, solvent-process) was a superior source of protein and that nutrients other than the amino acids were limiting in the casein diet and were contributed by the soybean meal. Moreover the soybean meal provided high levels of easily available potassium, magnesium, iron and copper. Considering these factors it was

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[3] Pleasants, J. R., D. R. Zimmerman, B. S. Reddy and B. S. Wostmann 1963 Rearing of gnotobiotic rabbits and guinea pigs. Proc. Gnotobiotic Workshop and Symposium. Ohio State University, p. 36.

decided to eliminate casein completely from the diet, provide more iron and copper from a natural source and to increase carbonate ions at the cost of sulfates. To this effect diet L-478 was formulated (table 1) which incorporated both soybean meal and a mineral mixture in which $MgSO_4$ was replaced largely by $MgCO_3$. The total levels of potassium, iron, copper and alfalfa meal were increased slightly over those in diet L-477.

The object of this investigation was to study the effect of this new diet L-478 in correcting the anemia and irregular pregnancies observed in germfree rabbits fed diets L-473E5 and L-477. The iron-transport system and iron and copper storage were also investigated since these 2 trace minerals are associated mainly with microcytic and hypochromic anemia in rabbits.

TABLE 1

Composition of solid diets developed for germfree rabbits

	Diets		
	L-473E5	L-477	L-478
	%	%	%
Wheat, ground	30	32	20
Corn, ground, yellow	9.25	14.55	15
Oats, rolled	20	22	18
Soybean meal	—	—	25
Lactalbumin	10	10	5
Casein	5	7.8	—
Whole milk powder	10	—	—
Alfalfa, dehydrated	2	2	5
Liver, desiccated	2	2	2
Corn oil	—	3	3
$CaCO_3$	1.1	1.1	0.75
$CaHPO_4 \cdot 2H_2O$	—	0.4	1
$Mg(C_2H_3O_2)_2 \cdot 4H_2O$	0.95	0.95	0.75
CH_3COOK	0.9	0.9	1.2
Salts-17 [1]	1.75	—	—
Salts-24 [2]	—	1	—
Salts-26 [3]	—	—	1
Ladek-3 (fat-soluble vitamins) [4]	2	2	2
B vitamin mixture-75 [4]	0.25	0.25	0.25
Inositol	0.05	0.05	0.05

[1] Salts-17 contained: (in milligrams) NaCl, 550; KI, 4.5; $MgSO_4$, 449; $FeC_6H_5O_7 \cdot 5H_2O$, 450; $CuSO_4 \cdot 5H_2O$, 22.4; $MnSO_4 \cdot H_2O$, 57.7; $ZnSO_4 \cdot H_2O$, 3.8; MoO_3, 3; NaF, 1.5; $CoCl_2 \cdot 6H_2O$, 3; $Na_2B_4O_7 \cdot 10H_2O$, 3; $AlK(SO_4)_2 \cdot 12H_2O$, 4.5.

[2] Salts-24 contained: (in milligrams) NaCl (iodized), 530; $MgSO_4$, 400; $FeC_6H_5O_7 \cdot 5H_2O$, 60; CuO, 2.5; $MnCO_3$, 4.2; ZnO, 2.5 and (in micrograms) MoO_3, 15; NaF, 22; $CoCl_2 \cdot 6H_2O$, 400; KBr, 10; Na_2SeO_3, 10.

[3] Salts-26 contained: (in milligrams) NaCl (iodized), 530; $MgSO_4$, 100; $MgCO_3$, 300; $FeC_6H_5O_7 \cdot 5H_2O$, 80; CuO, 3.8; $MnCO_3$, 4.2; ZnO, 2.5 and (in micrograms) MoO_3, 15; NaF, 22; $CoCl_2 \cdot 6H_2O$, 400; KBr, 10; Na_2SeO_3, 10.

[4] Zimmerman and Wostmann (3).

MATERIALS AND METHODS

The rabbits used in this investigation were of Dutch belted strain and mixed sex. The germfree rabbits were Caesarian-born and were handfed a milk diet by improved hand rearing methods (4). After weaning, the germfree rabbits were housed in Reyniers-type isolators with wire-screen bottoms (9) and maintained with either one of the steam sterilized diets L-473E5, L-477 or L-478 (table 1). Conventional and conventionalized [4] rabbits were housed in the temperature-controlled open colony room and fed steam-sterilized diets L-477 or L-478. The water content of all diets was approximately 30% before sterilization. The housing conditions of germfree and conventional animals were comparable.

Experiment 1. This preliminary experiment was designed to study the effects of protein and mineral composition of the diet and the influence of germfreeness on the blood hemoglobin levels and iron-transport system in adult rabbits. All animals were germfree and approximately 6 months old at the start of the experiment. Rabbits in group 1 had been maintained with diet L-473E5 from weaning, the animals in groups 2 and 3 had been fed diet L-477. At the start of the experiment the rabbits in group 1 were transferred to diet L-478, whereas animals in group 2 and 3 continued to be fed formula L-477. Groups 1 and 2 remained germfree, but the rabbits in group 3 were conventionalized by bringing them out of the germfree isolator to conventional animal quarters. The duration of the experiment was 6 weeks. Two animals were used in each group. Food and water were supplied ad libitum. Blood was collected from the heart into a heparinized syringe at the beginning of the experiment and also at weekly intervals. To avoid the effects of diurnal variation blood samples were taken at the same time of the day throughout the experimental period.

Experiment 2. Since experiment 1 had demonstrated that anemia in germfree rabbits fed diet L-473E5 or L-477 was alleviated either by transferring these animals to diet L-478 or by conventionalizing them, experiment 2 was designed to study the

[4] The expression "conventionalized" refers to germfree animals transferred from germfree isolators to conventional animal quarters where they could acquire a normal microbiota.

effect of continuous feeding of diet L-478 on hemoglobin levels, on the iron and copper transport systems, and on iron and copper storage. Five germfree and four conventional weanling rabbits were fed, ad libitum, diet L-478, and weekly weights were recorded. The duration of the experiment was 8 weeks. After the experimental period, the animals were anesthetized with ether, blood was collected from the heart into a heparinized syringe and the residual blood was removed as much as possible by bleeding freely from the carotid arteries. Liver, spleen and kidneys were removed, blotted gently to remove traces of blood and weighed. The weight of cecal contents was also determined, since even nutritionally adequate diets would not be useful if they caused abnormal cecal distention.[5]

Biochemical determinations. Iron and copper were determined in both steam-sterilized and nonsterilized diets by an AOAC colorimetric procedure (10). Methods for determining hemoglobin and hematocrit in blood, and for determining iron and copper in liver, spleen and kidney were as described previously (11). Plasma iron, total iron-binding capacity of plasma (TIBC) and percentage saturation of iron-binding protein in plasma were determined by the micromethod of Scarlata and Moore (12) and plasma copper by the method of Rice (13). Plasma protein was measured using the Atago refractometer.[6]

RESULTS

The iron and copper content of diets with and without added trace mineral supplement are presented in table 2. Comparison of the content before and after sterilization showed no demonstrable leaching of iron and copper during steam sterilization. The data show the high levels of iron and copper in diet L-473E5 resulting from mineral supplementation. Diet L-478, although containing much less total iron than diet L-473E5, contained almost 40% more iron and 3 to 4 times more copper in the natural ingredients of the diet than either diets L-473E5 or L-477.

The results of experiment 1 are shown in figures 1 and 2. Figure 1 shows that, at the start of the experiment, all hemoglobin and hematocrit levels were low, indicating a severe anemia in germfree rabbits maintained with diet L-473E5 or L-477. When the rabbits in group 1 were transferred from diet L-473E5 to L-478, the hemoglobin and hematocrit levels increased gradually and reached normal levels at the end of the fourth week. Germfree rabbits in group 2 maintained with diet L-477 showed the same low hemoglobin and hematocrit levels throughout the experimental period. However, when the animals in group 3 fed diet L-477 were conventionalized, the hemoglobin and hematocrit levels increased gradully to normal, the final values being slightly lower than the levels observed in germfree rabbits of group 1 after they were fed diet L-478 for 4 weeks.

Figure 2 shows the plasma copper, iron and total iron-binding capacity values of the rabbits in groups 1, 2 and 3. Plasma copper values were approximately similar in all groups at the start of the experiment. Although a slight increase in plasma copper levels was noticed in group 1 and 3 after the fourth week, the values from the start to the conclusion of the experiment were within normal range (14).

[5] See footnote 3.
[6] National Instrument Company Inc., Baltimore, Maryland.

TABLE 2
Iron and copper content of steam-sterilized and nonsterilized diets

	Diet L–473E5		Diet L–477		Diet L–478	
	NS [1]	SS [1]	NS	SS	NS	SS
	mg/100 g dry matter		*mg/100 g dry matter*		*mg/100 g dry matter*	
With trace mineral mixture						
Iron	86.5	86.2	15.9	15.8	23.3	23.1
Copper	6.93	6.90	2.90	2.91	5.97	5.99
Without trace mineral mixture						
Iron	5.01	5.03	5.00	4.98	8.81	8.75
Copper	0.73	0.72	0.73	0.71	2.69	2.63

[1] NS and SS indicate fresh nonsterilized and steam-sterilized diets, respectively.

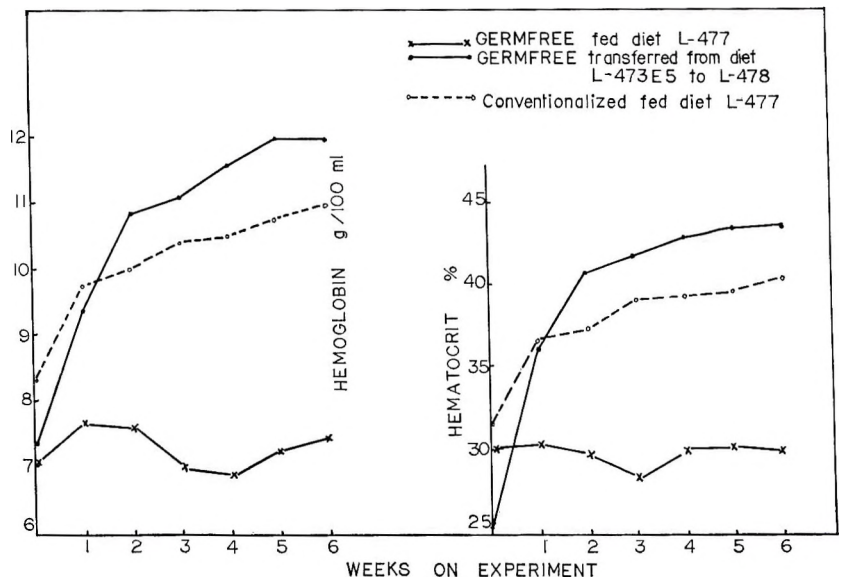

Fig. 1 Response of hemoglobin and hematocrit values in adult germfree rabbits to diet and to conventionalization (exp. 1).

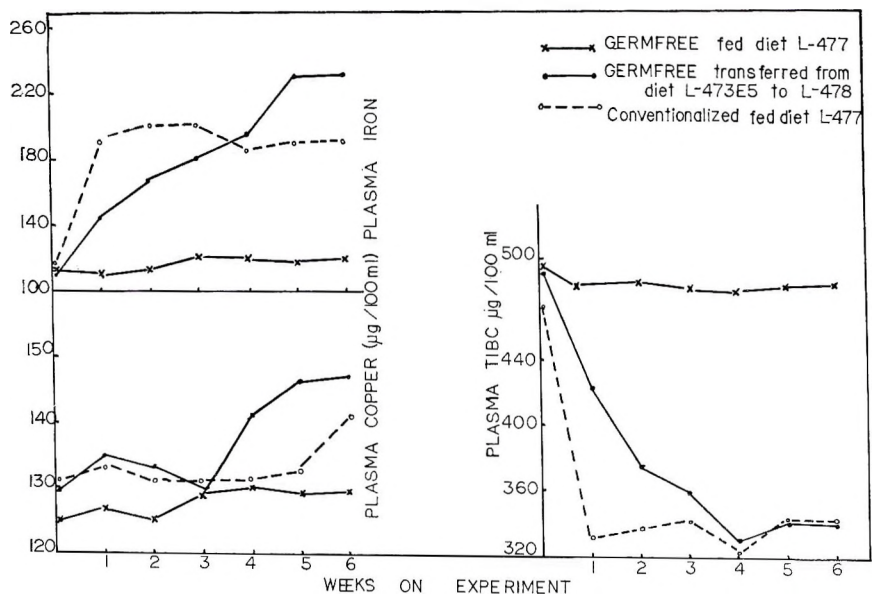

Fig. 2 Response of iron and copper transport-system in adult germfree rabbits to diet and to conventionalization (exp. 1).

Plasma iron levels were very low at the beginning of the experiment in rabbits fed either diet L-473E5 (group 1) or L-477 (groups 2 and 3). Germfree rabbits in group 1, when changed from diet L-473E5 to L-478, and the animals in group 3 after being conventionalized but continued with diet L-477, demonstrated an increase in plasma iron values to approximately normal levels during the experimental period. Germfree rabbits in group 2 fed diet L-477 continuously showed the same low levels throughout the experimental period. At the beginning of the experiment, the plasma TIBC of the germfree rabbits in groups 1, 2 and 3 was very high ranging from 470 to 496 μg/100 ml plasma. Following the change of diet for germfree rabbits in group 1 (L-473E5 to L-478), and also upon conventionalizing the germfree rabbits in group 3 (diet L-477), the TIBC values were changed to normal in about 4 weeks. No change in TIBC occurred in germfree rabbits of group 2 fed diet L-477 throughout.

The results of experiment 2 are shown in tables 3 and 4. Table 3 presents a summary of results on body and cecal content weights, and on blood constituents of germfree and conventional rabbits fed diet L-478. At the end of the experiment, there was no significant difference in body weights between germfree and conventional rabbits. Although the hemoglobin, hematocrit and plasma protein values in conventional animals were slightly but sig-

TABLE 3

Summary of observations on body and cecum weights and blood constituents of germfree and conventional rabbits fed diet L–478 (exp. 2)

	Germfree	Conventional
No. of animals	5	4
Body wt, g	1149 ± 108 [1]	1189 ± 122
Cecal contents, % body wt	11.5 ± 0.94 [2]	3.17 ± 0.10
Hemoglobin, g/100 ml	12.2 ± 0.08 [3]	13.0 ± 0.26
Hematocrit, %	43.1 ± 0.33 [4]	44.3 ± 0.28
Plasma constituents		
Protein, g/100 ml	5.22 ± 0.06 [3]	5.50 ± 0.07
Copper, μg/100 ml	143 ± 4.7	146 ± 3.3
Iron, μg/100 ml	213 ± 7.6	232 ± 5.8
TIBC,[5] μg/100 ml	342 ± 7.7	349 ± 6.4
Saturation, %	62.1 ± 1.75	65.3 ± 1.8

[1] Averages ± SE of mean.
[2] Difference from conventional significant, $P < 0.001$.
[3] Difference from conventional significant, $P < 0.025$.
[4] Difference from convetional significant, $P < 0.050$.
[5] TIBC indicates total iron-binding capacity.

TABLE 4

Iron and copper content of tissues of germfree and conventional rabbits fed diet L–478 [1] (exp. 2)

	Iron		Copper	
	Germfree	Conventional	Germfree	Conventional
	μg/g fresh tissue			
Liver	132 ± 3.6 [2,3]	161 ± 9.2	3.18 ± 0.09	3.27 ± 0.12
Spleen	95.9 ± 8.0 [4]	323 ± 21.2	1.15 ± 0.04	1.17 ± 0.05
Kidney	44.1 ± 2.8 [4]	75.5 ± 3.1	3.83 ± 0.06	4.00 ± 0.06
	μg/organ			
Liver	4906 ± 298 [5]	6774 ± 686	118 ± 7.3	137 ± 4.5
Spleen	29.3 ± 2.4 [4]	276 ± 28.1	0.35 ± 0.01 [4]	0.99 ± 0.07
Kidney	362 ± 24.3 [4]	876 ± 41.8	31.4 ± 3.0 [3]	46.8 ± 3.7

[1] Five germfree and 4 conventional rabbits.
[2] Averages ± SE of mean.
[3] Difference from conventional significant, $P < 0.025$.
[4] Difference from conventional significant, $P < 0.001$.
[5] Difference from conventional significant, $P < 0.050$.

nificantly higher than those of the germfree animals, the values in both groups were within the normal range. Slightly lower plasma protein levels in germfree rabbits can be ascribed to a low γ-globulin content (15). Cecal contents of germfree rabbits averaged 11.5% of body weight which is half of that previously reported (1). There was no significant difference in plasma copper, iron, TIBC and percentage saturation of iron-binding protein between germfree and conventional rabbits fed diet L-478. The present data for conventional rabbits are in agreement with the observations of Smith and Ellis (14) for plasma copper, and with Sheeler and Barber (16) for hemoglobin, hematocrit and plasma iron, TIBC and percentage saturation of iron-binding protein. The observation of normal hemoglobin, hematocrit and plasma iron and copper levels in germfree rabbits fed diet L-478 indicated the absence of any symptoms of anemia in constrast with the anemic conditions observed in the germfree animals maintained with diets L-473E5 and L-477. However, like formula L-478, these diets produced conventional animals with an apparently normal iron and copper status.

The iron and copper content of liver, spleen and kidney of germfree and conventional rabbits fed diet L-478 was recorded in table 4. Since neither our data nor the literature values showed evidence of sex difference in the tissue copper (18) of rabbits, the data from both sexes have been pooled. The iron content of liver, spleen and kidney, expressed both on a fresh tissue basis and on a total organ basis, was significantly lower in germfree rabbits than in conventional rabbits. However, there was no difference between germfree and conventional rabbits either in concentration or in content of copper in these organs with the exception of total kidney and spleen copper. This last difference could be attributed entirely to the difference in organ weights between the germfree and the conventional animal. The presence or absence of an intestinal microflora thus appears to have no influence on copper concentration in storage organs in contrast with its significant influence on iron concentration in those same organs.

Germfree rabbits maintained with diet L-478 appeared normal and healthy, and did not show symptoms of bone fragility or hind leg paralysis. So far, one germfree female fed diet L-478 has weaned three of four litters successfully, the fourth litter being lost through an accident in technique. One of its offspring has weaned a first litter successfully.

DISCUSSION

In germfree rabbits rendered anemic by diet L-473E5 or diet L-477 normal hemoglobin levels could be recovered either by the animals consuming a different type of diet L-478 or by their taking on a conventional intestinal microbiota (conventionalization). This latter result appears to agree with the reports that in rats (19) and in rabbits (20), alterations of the intestinal flora by antibiotic treatment were associated with a decrease in iron absorption. Since the amount of iron present in diets L-473E5 and L-477 was adequate for conventional rabbits, the availability of this iron must have been influenced by the presence of intestinal microorganisms. A recent investigation in our laboratory showed a more positive oxidation-reduction potential in the intestinal contents of the germfree rat as compared with the conventional animal.[7] Assuming that similar conditions exist in the germfree rabbit intestine, then the availability of the iron would possibly be affected by its valence state under the existing conditions.

The adequate availability of iron from diet L-478 for rabbits in both the germfree and conventional states cannot be ascribed to any one dietary feature since this diet differed from the other diets not only in the form and quantity of iron provided, but also in the protein components, in the cations present and in potentially iron-complexing materials (21). All of these factors could have an influence on the iron status of the animal. The ready availability of soybean meal iron has been demonstrated by Davis et al. (22) for chickens, and by Miller and Louis (23) for anemic rats. This factor appears, to the authors, to be the likeliest cause for the alleviation

[7] Wostmann, B. S., and E. Bruckner-Kardoss 1965 Oxidation-reduction potential of cecal contents of germfree and conventional rats. Federation Proc., 24: 203 (abstract).

of anemia in germfree rabbits changed from diet L-473E5 to diet L-478, but further investigation will be required to determine all the factors involved.

Germfree rabbits fed diet L-478 maintained normal levels of circulating iron and TIBC (table 3), yet stored less iron in the liver, speen and kidneys (table 4) than their conventional counterparts fed the same diet. Recent studies in our laboratory (24) have indicated that germfree rats contained approximately 22% less total blood volume and hemoglobin per 100 g body weight than conventional rats. If similar conditions should exist in germfree rabbits, the lower demand of the germfree animal for hemoglobin iron might account for the decreased storage of iron in the usual storage organs. Furthermore, the very low amount of iron present in the germfree rabbit spleen supports our observation with rats that the spleen is not a major iron storage organ in the germfree animal (11).

The ceca of the germfree rabbits fed diet L-478 contained 3.6 times as much contents as the ceca of their conventional counterparts. This ratio is considerably less than that reported on earlier types of diet (1) when the cecal contents constituted about 25% of the body weight of the germfree rabbit. With diet L-478 the contents amounted to 11.5%. A possible relationship of this reduction in cecal size to the improved iron status of the germfree rabbits fed L-478 was suggested by the work of McCall et al. (25) with conventional rats fed milk-base diets; supplementation with iron reduced the cecal size by approximately one-half. The reduced cecal size with diet L-478 made it unnecessary to carry out cecal ligation at weaning in order to prevent death by volvulus before maturity could be reached (4).

Diet L-478 has eliminated not only the symptoms of anemia but also the symptoms of bone fragility, hind leg paralysis, extreme cecal distention and irregular reproduction observed in germfree rabbits fed earlier diets. Further investigation would be required to determine how these symptoms were related to the anemia. It is concluded that the availability of iron to germfree rabbits is reduced by the absence of an intestinal microbiota, but that the resulting hypochromic and microcytic anemia can be prevented by providing iron in a form whose availability was not seriously affected by the germfree condition.

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Selected Hemocytological Effects of Vitamin B_6 Deficiency in Chicks [1,2]

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ABSTRACT One hundred and eight chicks were allotted at random to 3 treatments, with 3 replicates of each treatment and 12 chicks per replicate pen. Treatments were: 1) vitamin B_6-deficient diet (1.22 mg/kg) consumed ad libitum; 2) vitamin B_6-adequate diet (2.86 mg/kg) consumed ad libitum; and 3) vitamin B_6-adequate diet pair-fed to the amount consumed by the B_6-deficient chicks. During the 4-week experimental period, response was measured by hemoglobin, packed cell volume values, mortality, weight gains, and feed conversion ratios. Pair-fed chicks had significantly higher hemoglobin values than the vitamin B_6-deficient chicks in the second, third, and fourth weeks of the trial. Packed cell volume values of the pair-fed chicks were significantly higher than those of the vitamin B_6-deficient chicks in the second and fourth weeks of the trial. Chicks consuming the vitamin B_6-adequate diet ad libitum had hemoglobin and packed cell volume values which remained fairly constant throughout the 4-week experiment, at a level lower than those of the pair-fed chicks consuming the same vitamin B_6-adequate diet. Deaths observed in pair-fed chicks indicated that vitamin B_6 deficiency in chicks depressed appetite sufficiently that starvation was a factor in deaths observed in deficient chicks. Feed conversion ratios in deficient and pair-fed chicks were significantly poorer than those of the groups receiving adequate vitamin B_6 on an ad libitum feeding regimen. Weight gains of the deficient chicks and the pair-fed chicks were similar.

The essentiality of vitamin B_6 for chicks was recognized in 1939 when Hegsted et al. (1) and Jukes (2) reported on vitamin B_6 studies with chicks. These reports were followed by others detailing various aspects of vitamin B_6 metabolism in chicks. Jukes (2) reported no departures from the normal range in differential blood cell counts of chicks fed either a vitamin B_6-deficient basal diet or the basal diet supplemented with pyridoxine hydrochloride. These cell counts were made on the seventeenth day of the experimental period. Luckey et al. (3) reported that vitamin B_6 deficiency in chicks was accompanied by a definite anemia. Hegsted and Rao (4) reported microcytic anemia as one of the symptoms of vitamin B_6 deficiency in chicks. The present study was made to investigate further the relationship between vitamin B_6 and hemoglobin level and packed cell volume in chicks.

EXPERIMENTAL PROCEDURE

Broiler-type cockerels were used in the 4-week experiment. All chicks were housed in electrically heated battery-brooders for the duration of the experiment. After a one-week pre-trial period, during which all chicks consumed a commercial starter diet, experimental birds were allotted at random to treatments. The treatments were: 1) adequate vitamin B_6, consumption ad libitum, 2) deficient vitamin B_6, consumption ad libitum, and 3) adequate vitamin B_6, consumption restricted to that amount consumed by the deficient vitamin B_6 group within the same replication. The treatments are hereafter referred to as 1) Ad AL, 2) D AL, and 3) Ad R.

The diet contained the following ingredients: (in per cent) dextrose, 58.85; purified soybean protein, 28.00; soybean oil, 2.00; non-nutritive fiber, 3.00; vitamin premix, 1.00; mineral premix, 5.30; choline chloride (25%), 1.00; DL-methionine, 0.70; glycine, 0.10; and DL-tryptophan, 0.05. The vitamin premix contributed the following per kilogram of complete ration: vitamin A, 10,000 IU; vitamin D_3, 1,500 ICU; vitamin E, 22 IU; menadione, 4.4 mg; thiamine·HCl, 4.4 mg; inositol, 132 mg; *p*-aminobenzoic acid, 66 mg; niacin, 88

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[2] From a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, Iowa State University.

mg; ascorbic acid, 220 mg; vitamin B_{12}, 22 μg; and biotin, 220 μg.

The mineral premix added the following per kilogram of complete ration: (in grams) calcium, 11.7; phosphorus, 5.8; potassium, 2.16; sodium, 1.87; chlorine, 2.9 (and in milligrams) manganese, 286; magnesium, 0.9 g; zinc, 94.5; iron, 95; copper, 9.5; cobalt, 0.9; and iodine, 3.5. The adequate vitamin B_6 rations contained sufficient added pyridoxine·HCl to provide a level of 2.86 mg of vitamin B_6/kg of complete ration. This level had been shown previously to be an adequate vitamin B_6 level for broiler chicks (5). No pyridoxine·HCl was added to the deficient vitamin B_6 ration. The calculated vitamin B_6 level of this ration was 1.22 mg/kg. After mixing, all diets were stored in a walk-in cooler at $10° \pm 2°$.

The amount of feed consumed the previous day by each vitamin B_6-deficient group was determined each morning. This amount was then fed to the pair-fed group which received the Ad R ration. When mortality occurred in an experimental pen, mean feed consumption was determined for the D AL group, and appropriate adjustment made in the amount fed to the pair-fed group.

A completely random design was used for the experiment. Each treatment was applied to 3 replicate groups of 12 chicks per replicate. At the start of the trial, 6 chicks in each experimental pen were selected at random for use in hemoglobin (Hb) and packed cell volume (PCV) determinations. The same 6 chicks from each pen were used throughout the trial. If a death occurred, a replacement was selected at random from the other chicks in the respective pen.

Chicks were weighed and blood samples were obtained at weekly intervals. Free-flowing blood samples were obtained from wing vein for the Hb and PCV determinations. Hemoglobin determinations were made by using the technique of Bankowski (6) as modified by Denington and Lucas (7). Packed cell volume determinations were made by a microcapillary method similar to that described by Natelson (8).

Statistical analyses of these data included analyses of variance tests on weekly Hb level, weekly PCV values, experimental period weight gains, and experimental period feed conversion ratios and determination of the correlations between Hb and PCV values within treatments. Two planned comparisons were made on the data in each analysis of variance test. First, data from chicks consuming the Ad R diet were compared with that of chicks consuming the D AL diet. Second, data from chicks consuming the Ad AL diet were compared with the combined data from chicks consuming the other 2 rations.

All statements concerning statistical significance are made at probability of 0.05 or less.

RESULTS AND DISCUSSION

Results of the analysis of variance tests on hemoglobin values indicated that chicks fed the vitamin B_6-deficient diet (1.22 mg/kg) had hemoglobin values which were significantly depressed compared with those of the pair-fed groups in the second, third, and fourth weeks of the trial (table 1, comparison 1 and fig. 1). When the Hb values for the these 2 groups were combined and compared with the groups receiving adequate vitamin B_6 on an ad libitum feeding regimen, the mean hemoglobin value for the combined groups was significantly higher at the end of the first and third weeks (table 1, comparison 2). The data from the second week approached significance. Luckey et al. (3) had observed a lower Hb level in vitamin B_6-deficient chicks than in chicks receiving a complete ration.

These results indicate that vitamin B_6-deficient chicks become anemic due to a metabolic failure in their normal pathway for hemoglobin synthesis and degradation. Since the deficient chicks had significantly depressed values over the pair-fed group, this failure is not due to the reduced feed intake per se, but results from the insufficient intake of vitamin B_6. The reduced feed intake (Ad R) resulted in hemoglobin values higher than those observed in chicks receiving adequate vitamin B_6 on an ad libitum feeding regimen (fig. 1). The noticeable increase in Hb level observed in chicks fed the Ad R ration over the level of those consuming the Ad AL ration may have been related to a Hb concentration occurring in the chicks whose growth was

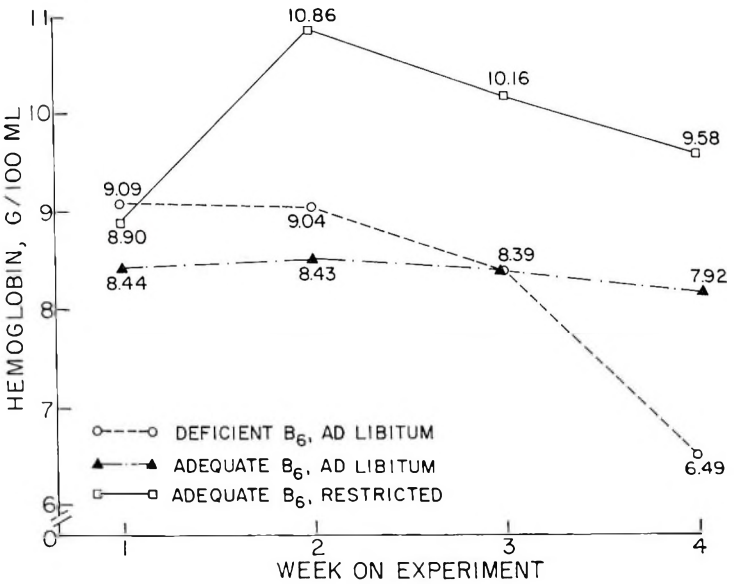

Fig. 1 Effect of feeding regimen on hemoglobin level.

TABLE 1

Summary of mean squares from analyses of variance tests on weekly hemoglobin and packed cell volume values

Determination and source of variation	df	Week of experiment			
		First	Second	Third	Fourth
Hemoglobin					
Comparison 1 [1]	1	0.0541	4.9504*	4.7171**	14.2913**
Comparison 2 [2]	1	0.6087*	4.6309	4.7432**	0.0242
Error	6	0.0952	0.8176	0.2673	1.0388
Packed cell volume					
Comparison 1	1	3.53	37.50*	32.67	150.00*
Comparison 2	1	13.52*	44.18**	50.67	6.97
Error	6	1.37	2.96	10.84	15.39

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
[1] Comparison made between groups fed deficient vitamin B_6, consumption ad libitum (D AL) and groups fed adequate vitamin B_6, with consumption restricted to that amount consumed by the deficient vitamin B_6 group within the same replication (Ad R).
[2] Comparison between group receiving adequate vitamin B_6, consumption ad libitum (Ad AL) and combined value of groups fed Ad R and D AL.

severely restricted by the limited amount of feed they were allowed to consume.

The individual PCV determinations within a treatment were more variable than the Hb determinations. However, the analysis of variance tests showed a generally similar pattern (table 1). The PCV values of chicks consuming the D AL ration exhibited a nearly linear decrease with time over the period studied in this experiment (fig. 2). A decrease was also noted in chicks fed the Ad AL ration, but this decrease was smaller and appeared to have reached a plateau at the termination of the trial.

Restricting the feed intake of the pair-fed chicks resulted in feed conversion values similar to those of chicks consuming the D AL ration (table 2). Whereas differences existed, these were not statistically significant. The group receiving adequate vitamin B_6 with ad libitum intake had a significantly better feed conversion than that of the other 2 groups.

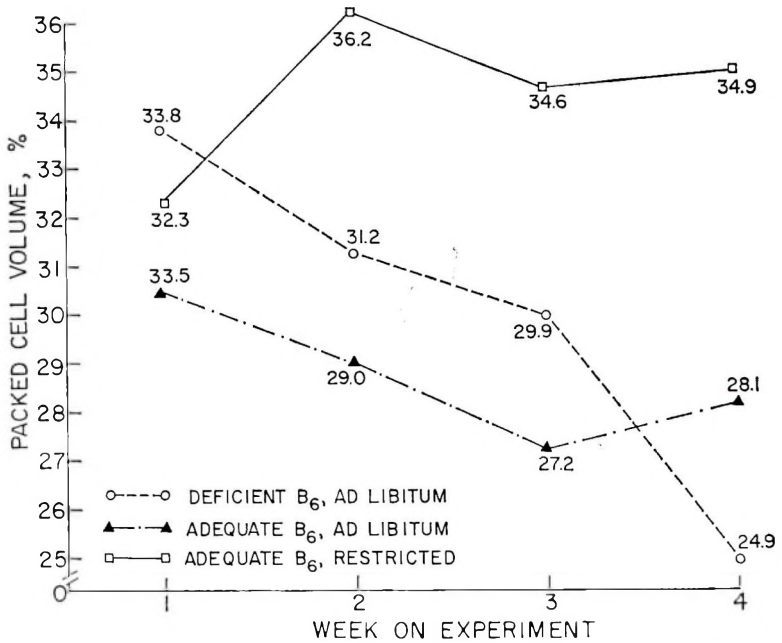

Fig. 2 Effect of feeding regimen on packed cell volume.

TABLE 2

Average weight gains, feed conversion ratios, and mortality

Feeding regimen	4-Week wt gains of survivors [1]	Feed conversion ratios (F/G)	4-Week mortality
	g		%
Deficient vitamin B_6, ad libitum intake	55.7 ± 6.39 [2]	4.02 ± 0.153	80.5 ± 4.79
Adequate vitamin B_6, restricted intake	62.6 ± 5.67	4.45 ± 0.207	27.8 ± 9.58
Adequate vitamin B_6, ad libitum intake	415.9 ± 5.24	2.03 ± 0.038	2.8 ± 4.79

[1] All pens contained 12 chicks initially.
[2] Mean ± SE of mean.

Weight gains of chicks receiving the same amount of feed were similar (table 2), indicating that a major cause of the greatly reduced rate of gain in vitamin B_6-deficient chicks is the loss of appetite, associated with a vitamin B_6 deficiency, and consequent reduced feed intake. Most researchers report loss of appetite as one of the vitamin B_6-deficiency symptoms in chicks. Weight gains of chicks receiving the Ad AL diet were about 7 times greater than gains of chicks consuming either the Ad R or D AL diets.

Mortality data (table 2) indicate that some deaths observed in vitamin B_6-deficient chicks were due to starvation mediated through loss of appetite rather than to direct metabolic effects such as loss of co-enzyme activity. Most reports indicate that deaths of chicks receiving vitamin B_6-deficient diets occur at about 10 to 14 days after the chicks start to eat the vitamin B_6-deficient diet. In this trial, nearly one-third of the deaths of chicks receiving deficient diets and one-fifth of the deaths of chicks receiving adequate vitamin B_6 at a restricted intake occurred in the tenth to fourteenth day of depletion. The one death recorded in the Ad AL groups occurred in this period. Postmortem observations of deficient chicks showed varying degrees of wing feather follicle hemorrhage, whereas

none was observed in chicks fed diets containing adequate vitamin B_6. Daghir and Balloun (5) reported wing feather follicle hemorrhage as one of the symptoms of vitamin B_6-deficient chicks.

Correlation coefficients obtained between PCV and Hb within the different treatments were 0.914, 0.768, and 0.752 for D AL, Ad R, and Ad AL, respectively. The test for homogeneity of these coefficients indicated that they were homogeneous, and a pooled correlation coefficient was determined. This value of 0.829 was statistically significant.

The extent to which weight gains of chicks receiving the adequate vitamin B_6 diet in restricted amounts were depressed and the number of deaths observed in these chicks were unexpected. These results show the importance of vitamin B_6 in stimulating chicks to eat. The means through which vitamin B_6 enhances appetite is unknown.

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Influence of Carbohydrate-to-Fat Ratio on Metabolic Changes Induced in Rats by Feeding Different Carbohydrate-Fat Combinations [1]

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ABSTRACT This study was designed to determine whether changing the relative proportions of carbohydrate and fat in the diet would influence metabolic responses in rats to different sources of the 2 nutrients. Four carbohydrate-fat combinations (glucose and fructose each with corn oil (CO) and with hydrogenated coconut oil (HCO)) were combined in high carbohydrate:low fat and low carbohydrate:high fat diets. Protein and caloric values of all diets were equivalent. Eight groups of male, weanling rats were each fed one of the experimental diets for 2 to 4 weeks. Reducing the carbohydrate-to-fat ratio from 64:5 to 19:25 (by weight) resulted in the following changes in liver functions: 1) marked reduction or complete elimination of responses of the glucose-6-phosphatase and fructose diphosphatase enzyme systems to dietary fructose; 2) significant increase in response of glucose-6-phosphatase to dietary HCO; 3) decreases in liver glycogen, to a different extent with different carbohydrate-fat combinations; 4) striking increases in total lipid in rats fed CO or HCO with glucose; and 5) increases in cholesterol in rats fed CO, and in phospholipid in rats fed HCO.

Previous work from this laboratory (1, 2) demonstrated that metabolic responses in rats to different types of dietary carbohydrate can be modified by varying the type of dietary fat. Conversely, effects on liver lipids of substituting one fat source for another depend, in part, on the carbohydrate component of the diet.

The present study was designed to observe any influence on these interrelationships of altering the relative proportions of carbohydrate and fat in the diet. Carbohydrate-fat combinations identical with those used in one of the above studies (1) were fed in 2 ratios: high carbohydrate-to-low fat and low carbohydrate-to-high fat. Livers were assayed, as before, for activities of 2 glycolytic enzyme systems and for content of lipids and glycogen, to detect concurrent effects on carbohydrate and lipid metabolism induced by the various diets.

EXPERIMENTAL

Eight groups of 12 male, weanling, albino rats of the Sprague-Dawley strain [2] were housed individually in mesh-bottom cages in an air conditioned laboratory, and given water and food ad libitum for 2 to 4 weeks. All rations contained: (in per cent) protein, 20 (casein supplemented with 2% of DL-methionine); salts,[3] 4; vitamin mix,[4] 0.25; and choline chloride, 0.2. The remainder of each ration consisted of carbohydrate, fat, and non-nutritive bulk,[5] in quantities calculated to make rations approximately equicaloric (380 kcal/100 g ration). Carbohydrate-fat combinations were glucose with corn oil [6] (G-CO), glucose with hydrogenated coconut oil [7] (G-HCO), fructose with corn oil (F-CO), and fructose with hydrogenated coconut oil (F-HCO). Each combination was incorporated into diets in 2 ratios: (per cent of total diet) carbohydrate, 64 with fat, 5 (HC:LF); and carbohydrate, 19 with fat, 25 (LC:HF).

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[2] Obtained from Hormone Assay Laboratories, Inc., Chicago.
[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.
[4] The vitamin mixture provided the following: (in mg/100 g ration) thiamine·HCl, 0.8; riboflavin, 0.6; pyridoxine, 0.4; Ca pantothenate, 4.0; niacin, 5.0; inositol, 20.0; folic acid, 0.4; vitamin B_{12}, 0.004; biotin, 0.02; vitamin A powder, 10.0 (200 units); calciferol, 0.18 (150 units); DL-α-tocopherol powder, 30.0 (7.5 units); and menadione, 0.38.
[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[6] Mazola, Corn Products Company, Argo, Illinois.
[7] Hydrol, Durkee Famous Foods, Chicago.

One-half of each group (total of 48 rats) was killed approximately 2 weeks (days 12 through 17), and half after approximately 4 weeks (days 26 through 31). The 2 periods were chosen to cover times of maximal effect of these carbohydrate-fat combinations on enzyme activities (2 weeks) and lipid levels (4 weeks) observed previously (1,2). A similar time relationship, enzyme changes before maximal lipid deposition, occurs also in livers of young rats fed different types of diets (3, 4). To avoid decreases in liver glycogen associated with the post-absorptive state, all animals were killed early in the morning, when stomachs still contained substantial amounts of food. Because of this time restriction, and the number of determinations made on each liver, only 8 rats (1/group) could be killed per day, necessitating the 6-day periods. Since one rat from each group was killed daily, any changes with time during the 6 days would tend to increase standard errors within groups, but should not affect relationships among groups.

After rats had been fed experimental diets for the times specified, they were decapitated. Livers were removed rapidly, chilled, homogenized, and assayed for activities of the glucose-6-phosphatase (G-6-Pase) and fructose diphosphatase (FDPase) enzyme systems, glycogen, and labile phosphorus from ADP + ATP. The remainder of each homogenate was stored frozen and analyzed later for total lipid, phospholipid, and cholesterol. Methods for the above assays have been described previously (1, 2). Nitrogen was determined in dried, ground, fat-free samples by the Dumas method.[8]

RESULTS

Growth rates and food intakes

For the first 2-week period, average weekly weight gains were comparable for all groups except the two receiving diets containing 25% of HCO (LC:HF), in which rate of gain was depressed by al-

[8] Coleman Nitrogen Analyzer, Model 29.

TABLE 1

Weight gains, food intakes, and food efficiency ratios of rats fed different carbohydrate-fat combinations in 2 ratios

C:F [1] ratio	Weeks fed diets	No. rats/ group	G–CO	F–CO	G–HCO	F–HCO
			Weight gain, g/week			
HC:LF [2]	2	12	35 ± 1 [3]	34 ± 2	34 ± 2	31 ± 1
HC:LF	4	6	35 ± 4	30 ± 2	33 ± 2	25 ± 2
LC:HF [2]	2	12	35 ± 1	35 ± 1	25 ± 2	25 ± 1
LC:HF	4	6	40 ± 1	31 ± 3	27 ± 1	25 ± 1
			Food intake, g/week			
HC:LF	2	12	70 ± 2	65 ± 3	68 ± 3	67 ± 2
HC:LF	4	6	105 ± 7	92 ± 7	104 ± 7	94 ± 5
LC:HF	2	12	72 ± 1	67 ± 2	64 ± 2	60 ± 2
LC:HF	4	6	107 ± 3	91 ± 5	92 ± 3	81 ± 5
			Food efficiency ratios [4]			
HC:LF	2	12	50 ± 1	52 ± 1	51 ± 1	47 ± 1
HC:LF	4	6	33 ± 2	33 ± 1	31 ± 1	26 ± 1
LC:HF	2	12	48 ± 1	51 ± 1	39 ± 2	42 ± 5
LC:HF	4	6	37 ± 1	34 ± 2	28 ± 1	30 ± 1
			Final body weights, g			
HC:LF	2	12	129 ± 4	127 ± 6	124 ± 4	116 ± 3
HC:LF	4	6	184 ± 12	169 ± 11	176 ± 12	151 ± 4
LC:HF	2	12	127 ± 6	121 ± 3	93 ± 17	103 ± 4
LC:HF	4	6	198 ± 5	177 ± 11	149 ± 4	138 ± 4

[1] Abbreviations: C:F ratio = carbohydrate:fat ratio, HC:LF = high carbohydrate:low fat diets, etc.; G = glucose, F = fructose, CO = corn oil, HCO = hydrogenated coconut oil.
[2] HC:LF = 64:5, LC:HF = 19:25.
[3] Averages ± SE of mean.
[4] Weight gain/food intake x 100.

most 30% (table 1). During the second 2-week period, rate of gain was not further depressed in these 2 groups, but a similar depression occurred in the group receiving fructose with 5% of HCO (HC:LF). Examination of food intake data and food efficiency ratios (table 1) indicates that the lower rates of weight gain can be attributed primarily to less efficient utilization of the respective diets. The poorer utilization of HCO is apparently not due to lack of absorption of the hydrogenated fat, because metabolic responses to HCO differ markedly from those to a fat-free diet in rats gaining at the same rate.[9] Average final body weights of rats fed fructose with 5% of HCO, glucose with 25% of HCO, or fructose with 25% of HCO were lower than the average weight of control rats (G with 5% of CO) by 18, 19, and 25%, respectively.

Relative size and composition of livers

Average relative weights of livers and content of major constituents (g/100 g body weight) for each group at 2 weeks are illustrated graphically in figure 1a, and percentage composition of liver with respect to protein, glycogen, and total lipid in figure 1b. Substitution of fructose for glucose in HC:LF diets containing either CO or HCO resulted in increases of approximately 30% in relative liver weights ($P < 0.01$).[10] This was not unexpected, since a stimulating effect of dietary fructose on liver size has been observed frequently (1, 2, 5, 6). However, the same substitution in LC:HF diets resulted in no significant increase in liver size. Among groups fed LC:HF diets, the group fed 25% of HCO with glucose had the largest livers in

[9] Unpublished data.
[10] Student's t test.

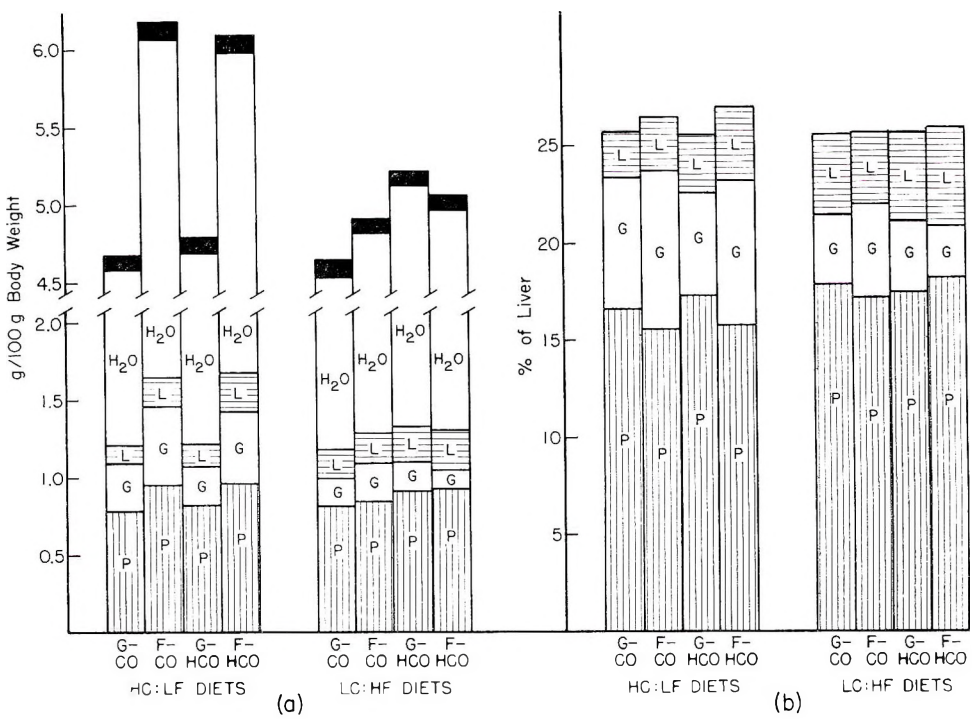

Fig. 1 Relative weights and composition of livers from rats fed different carbohydrate-fat combinations in 2 ratios for 2 weeks. (a) Grams of total liver and major constituents per 100 g body weight. (b) Per cent composition of liver with respect to protein, glycogen, and total lipid. HC:LF = high carbohydrate:low fat, LC:HF = low carbohydrate-high fat, G-CO = glucose with corn oil, F-CO = fructose with corn oil, G-HCO = glucose with hydrogenated coconut oil, F-HCO-fructose with hydrogenated coconut oil, P = protein, G = glycogen, and L = lipid.

relation to body weight, 13% heavier ($P < 0.01$) than those from groups fed 25% of CO with glucose.

The increase in total protein (fig. 1a) in livers from rats fed the high fructose diets fell somewhat behind the increase in total liver tissue, with the result that the percentage of protein in these livers (fig. 1b) was slightly less than that in livers from corresponding groups fed glucose ($P < 0.01$ with HCO diets). Nevertheless, increases in glycogen, and to a lesser extent in lipid, were such that percentages as well as total amounts of these constituents were greater in the larger livers. Enlargement of livers of rats fed 25% of HCO with glucose was accompanied by a 30% increase in total lipid, but no change in glycogen, as compared with the corresponding group fed CO. These differences in liver composition indicate that although relative liver size increased in response to high dietary levels of either fructose or HCO, metabolic changes reflected in each case were different. Average moisture content of livers from all groups was 71 or 72%.

Liver lipids

Liver lipid data are expressed as milligrams per 100 mg of liver nitrogen (table 2). Since values for all except one group were similar at 2 and 4 weeks, data from the 2 time periods were combined. The exception was the group fed glucose with 25% of CO, in which total lipid values increased from 143 ± 13 at 2 weeks to 194 ±13 at 4 weeks, phospholipid from 20 ± 1 to 32 ± 6, and cholesterol from 24 ± 2 to 30 ± 3.

In general, livers from rats fed LC:HF diets contained more lipid in relation to amount of liver nitrogen than did livers from rats fed HC:LF diets. However, there was no direct correlation between amount of dietary fat and amount of liver lipid, since the combination of carbohydrate and fat fed also had significant effects on degree of lipid accumulation. Decreasing the C:F ratio of diets containing glucose resulted in highly significant increases ($P < 0.01$) in total liver lipid, but the C:F ratio had little effect on level of liver lipid in rats fed diets containing fructose.

Both the type of carbohydrate and the type of fat in HC:LF diets significantly influenced lipid content of the liver. Substitution of fructose for glucose with CO enhanced lipid deposition by 19% ($P < 0.05$), and with HCO by 35% ($P < 0.01$), whereas livers from rats fed HCO with either carbohydrate contained more lipid ($P < 0.01$) than did livers from corresponding groups fed CO. The striking increase in liver lipid in the F-HCO group suggests a synergistic effect of fructose and HCO. When the 4 carbohydrate-fat combinations were fed in LC:HF diets, degree of liver lipid accumulation was comparable in all groups except the F-CO group, in which

TABLE 2

Total lipid, phospholipid, and cholesterol in livers from rats fed different carbohydrate-fat combinations in 2 ratios for 2 to 4 weeks [1]

C:F [2] ratio	No. rats/ group	G–CO	F–CO	G–HCO	F–HCO
		mg/100 mg liver nitrogen			
		Total liver lipid			
HC:LF [3]	12	96 ± 5 [4]	114 ± 7	115 ± 4	155 ± 7
LC:HF [3]	12	168 ± 12	134 ± 7	177 ± 8	168 ± 6
		Liver phospholipid			
HC:LF	12	18 ± 3	23 ± 2	33 ± 3	40 ± 2
LC:HF	12	26 ± 3	21 ± 2	46 ± 4	55 ± 3
		Liver cholesterol			
HC:LF	12	16 ± 1	15 ± 1	16 ± 1	16 ± 1
LC:HF	12	27 ± 2	23 ± 1	17 ± 1	17 ± 1

[1] Data for 2- and 4-week periods combined.
[2] Abbreviations: C:F ratio = carbohydrate:fat ratio, HC:LF = high carbohydrate:low fat diets, etc.; G = glucose, F = fructose, CO = corn oil, HCO = hydrogenated coconut oil.
[3] HC:LF = 64:5, LC:HF = 19:25.
[4] Averages ± SE of mean.

amount of lipid was 20% less ($P < 0.05$) than that in the G-CO group.

The amount of phospholipid in the livers was influenced more consistently by the HCO content of the diet than by any other factor (table 2). Substituting HCO for CO, or increasing HCO from 5 to 25% of the diet, with either carbohydrate, resulted in significant increases in liver phospholipid. All differences between values for HCO-fed groups and the corresponding CO-fed groups were significant at the 1% level; and effects of increasing the proportion of HCO with glucose was significant at the 2% level, and with fructose at the 1% level. The greatest amount of phospholipid was noted in livers from rats fed the high HCO diet with fructose.

Cholesterol content of liver responded to the level of dietary CO. Cholesterol level was not influenced by type of dietary fat at the 5% level, but increasing CO from 5 to 25% in diets containing either glucose or fructose resulted in substantial increases in liver cholesterol, significant at the 1% level. Liver cholesterol levels in the other 6 groups were identical, despite wide variations in total lipid (table 2).

Enzyme activities, glycogen, nitrogen, and labile phosphorus from ADP + ATP

Activities of the G-6-Pase and FDPase enzyme systems are expressed as units per 100 g of body weight, and glycogen as milligrams per 100 g of body weight (table 3). As discussed in a previous paper (2), body weight was considered a more meaningful basis than liver weight or liver nitrogen for evaluating differences in these

TABLE 3

Activities of the G-6-Pase and FDPase enzyme systems, glycogen, nitrogen, and labile phosphorus in livers from rats fed different carbohydrate-fat combinations in 2 ratios

C:F [1] ratio	Weeks fed diets	No. rats/ group	G–CO	F–CO	G–HCO	F–HCO
			G-6-Pase activity, units [2]/100 g body weight			
HC:LF [3]	2	6	93 ± 1 [4]	182 ± 9	116 ± 5	182 ± 7
HC:LF	4	6	98 ± 4	158 ± 7	103 ± 4	168 ± 8
LC:HF [3]	2	6	92 ± 5	116 ± 9	142 ± 6	153 ± 4
LC:HF	4	6	88 ± 4	102 ± 7	147 ± 3	161 ± 6
			FDPase activity, units/100 g body weight			
HC:LF	2	6	50 ± 2	77 ± 3	47 ± 2	60 ± 3
HC:LF	4	6	51 ± 1	70 ± 3	44 ± 3	58 ± 5
LC:HF	2	6	60 ± 4	61 ± 2	59 ± 1	62 ± 3
LC:HF	4	6	46 ± 2	54 ± 2	60 ± 4	59 ± 3
			Glycogen, mg/100 g body weight			
HC:LF	2	6	318 ± 21	512 ± 49	261 ± 23	455 ± 37
HC:LF	4	6	340 ± 27	399 ± 24	172 ± 19	327 ± 30
LC:HF	2	6	168 ± 8	232 ± 15	186 ± 11	138 ± 22
LC:HF	4	6	177 ± 15	273 ± 32	137 ± 15	152 ± 6
			Nitrogen, mg/100 g body weight			
HC:LF	2	6	125 ± 3	153 ± 2	131 ± 3	155 ± 5
HC:LF	4	6	119 ± 3	147 ± 3	121 ± 4	147 ± 3
LC:HF	2	6	133 ± 4	138 ± 5	146 ± 3	147 ± 2
LC:HF	4	6	125 ± 1	128 ± 4	158 ± 5	155 ± 4
			Labile phosphorus from ADP and ATP, μg/100 mg liver nitrogen			
HC:LF	2	6	251 ± 15	336 ± 22	233 ± 15	275 ± 21
HC:LF	4	6	174 ± 23	223 ± 28	156 ± 4	188 ± 15
LC:HF	2	6	250 ± 32	304 ± 26	195 ± 15	222 ± 17
LC:HF	4	6	198 ± 18	209 ± 16	147 ± 11	152 ± 10

[1] Abbreviations: C:F ratio = carbohydrate:fat ratio, HC:LF = high carbohydrate: low fat diets, etc.; G = glucose, F = fructose, CO = corn oil, HCO = hydrogenated coconut oil; G-6-Pase = glucose-6-phosphatase, FDPase = fructose-1,6-diphosphatase.

[2] One unit = activity catalyzing release of 1 μmole of inorganic phosphorus/minute, at 37.5°.

[3] HC:LF = 64:5, LC:HF = 19:25.

[4] Averages ± SE of mean.

functions associated with supply of glucose to all body tissues.

Substituting fructose for glucose in HC:LF diets containing CO resulted in an approximate doubling of G-6-Pase activity at 2 weeks. Activity in livers from rats fed glucose with 5% of HCO was 25% higher ($P < 0.01$) than that in livers from rats fed glucose with 5% of CO. When fructose and HCO were fed together in a HC:LF diet, activity was identical with that for the group fed fructose with CO, indicating that effects of fructose and HCO were not additive. Thus, substitution of fructose for glucose in diets containing HCO resulted in a stimulation of G-6-Pase activity of only 57% ($P < 0.01$) as compared with the almost 100% increase induced by substitution of fructose for glucose in CO diets (above). At 4 weeks, differences between values for the control group and the 2 groups fed fructose had decreased somewhat, and stimulation by the G-HCO diet was no longer evident.

When the ratio of C:F in the diets was decreased (LC:HF diets), activity in the control group (G-CO) was essentially the same as that in the corresponding HC:LF control group, but activities in the other 3 groups, and relationships among groups, were substantially altered. The most marked differences were the much smaller degree of stimulation (only 26%) by fructose in CO diets ($P < 0.05$), and the much greater degree of stimulation (54%) by HCO fed with glucose ($P < 0.01$). Again, effects of fructose and HCO did not appear to be additive. At 4 weeks, activities in livers from all groups fed the LC:HF diets remained essentially the same as at 2 weeks. Figure 2 illustrates the striking effect of altering the C:F ratio of the diet on the relative degrees of response of G-6-Pase to different C-F combinations. Values from rats fed 15% fat diet [11] under comparable laboratory conditions are presented for comparison.

[11] Unpublished experiment.

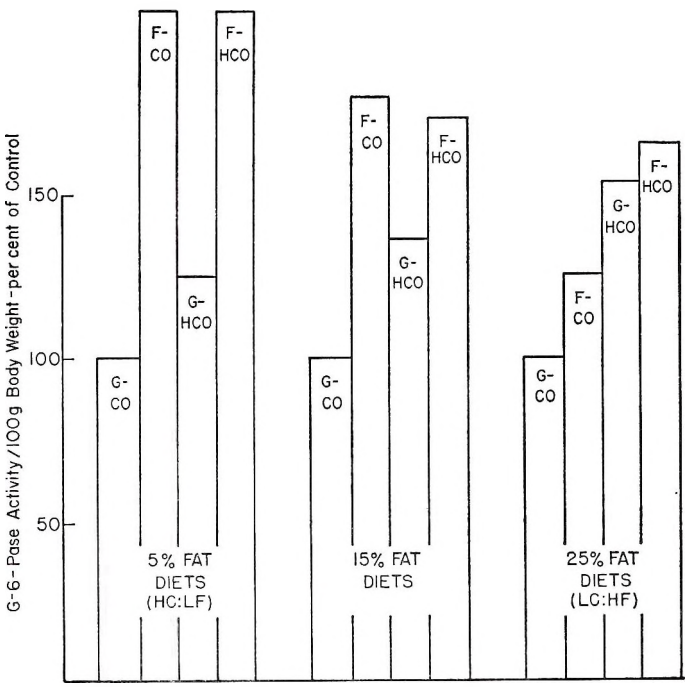

Fig. 2 Effect of proportions of fat in the diet on responses of G-6-Pase activity to different carbohydrate-fat combinations. Each bar represents data from 6 rats. HC:LF = high carbohydrate:low fat diets and LC:HF = low carbohydrate:high fat diets, containing 5 and 25% of fat, respectively. Data for 15% fat diets are from an unpublished experiment. G = glucose, F = fructose, CO = corn oil, and HCO = hydrogenated coconut oil.

With diets used in previous experiments (1, 2), responses of the FDPase enzyme system to various C-F combinations differed from those of the G-6-Pase system. The same was true in this study (table 3). FDPase activity in livers from rats fed fructose with 5% of CO was significantly greater ($P < 0.01$) than that from rats fed the corresponding glucose diet, but the percentage increase at 2 weeks was only about half that observed in G-6-Pase activity in the same rats. Moreover, unlike G-6-Pase activity, FDPase activity was not stimulated by dietary HCO, as evidenced by essentially equal values for the groups fed the G-CO and G-HCO combinations in HC:LF diets at 2 weeks, and the relative depression in FDPase activity in this G-HCO group at 4 weeks ($P < 0.05$). Increasing the proportion of either CO or HCO (LC:HF vs. HC:LF) resulted in significantly greater FDPase activity in livers from rats fed glucose (with CO, $P < 0.05$, with HCO, $P < 0.01$), but eliminated, or masked, the stimulating effect of fructose, so that activities of the 4 groups fed the LC:HF diets were identical at 2 weeks. By 4 weeks, values for the 2 groups receiving 25% of CO had decreased to levels approximating that of the HC:LF control group, whereas values for the 2 groups receiving 25% of HCO remained elevated.

Glycogen content of livers was especially sensitive to differences in both C:F ratios and C-F combinations in the diets (table 3). Values for all groups fed LC:HF diets were significantly lower than those for corresponding groups fed HC:LF diets, at both 2 and 4 weeks. However, there were major differences among groups fed diets containing the same C:F ratio. An enhancement of net glycogen storage in response to dietary fructose, observed previously with 15% fat diets (2), was again apparent with HC:LF diets containing either CO or HCO at 2 weeks ($P < 0.01$). This effect was also observed with the substitution of fructose for glucose in the LF:HC diet containing CO ($P < 0.01$), but not in that containing HCO. At 4 weeks, values for the 2 control groups (G-CO) had not changed, but large accumulations of glycogen in livers of rats fed diets high in fructose had decreased considerably, and glycogen content of livers from rats fed glucose with 5% of HCO had decreased by 55% ($P < 0.05$) from the 2-week level.

Values for total liver nitrogen are included in table 3 so that changes in specific functions may be compared with relative amounts of "active" liver tissue in response to the various diets.

Labile phosphorus is expressed as micrograms per 100 mg of liver nitrogen (table 3). This appeared to be the most reasonable basis for evaluating differences in ADP + ATP, since their utilization is closely linked with reactions of enzymes, the major protein constituents of liver cells. Substitution of fructose for glucose in the HC:LF diet containing CO resulted in a 34% increase in labile phosphorus at 2 weeks ($P < 0.02$). However, when HCO was the dietary fat, this effect of fructose was barely evident. Decreasing the C:F ratio of the F-CO diet tended to reduce the influence of dietary fructose, and because of large variations within groups fed LC:HF diets, the difference between F-CO and G-CO groups was not significant. By 4 weeks, labile phosphorus content of livers from the 2 groups fed diets containing 25% of HCO had decreased by about 25% below corresponding values for the groups fed 25% of CO. Differences were significant at the 5% level for both G-HCO vs. G-CO and F-HCO vs. F-CO. Assuming that labile phosphorus values reflect ATP levels (7), these results would indicate that dietary fructose tends to increase, and dietary HCO to decrease, the net amount of ATP in liver tissue. Understandably, effects of fructose were most evident with HC:LF diets, and those of HCO with LC:HF diets. Similar influences of fructose and saturated fat on labile phosphorus from ADP + ATP were reported and discussed previously (2).

DISCUSSION

Results from this study demonstrate that changing relative proportions of carbohydrate and fat in the diet can modify metabolic responses to different carbohydrate-fat combinations. This is strikingly illustrated in degrees of lipid accumulation in response to different ratios of the same carbohydrate-fat combination (table 2). The difference of 75% in total liver lipid between groups fed 5 and 25% of CO

with glucose, together with the accumulation of cholesterol in livers of both groups fed 25% of CO, strongly suggests a derangement of lipid metabolism resulting from the high dietary level of CO. Others have also observed increasing amounts of liver total lipid (8) and cholesterol (8–10), mainly in the esterified fraction, with increasing amounts of dietary polyunsaturated fatty acids (PUFA) above the level required to prevent essential fatty acid (EFA) deficiency. It is possible that changes in lipid metabolism in response to high intake of CO could be associated with a relative excess of PUFA in liver, due to both the large amount of linoleate ingested, and inhibition of synthesis of monounsaturated and saturated fatty acids associated with high dietary levels of long-chain fatty acids (8, 12–13). The greater amount of liver lipid in the group fed glucose with 25% of HCO as compared with 5% of HCO can probably be attributed to increased requirement for essential fatty acids with the higher dietary level of saturated fat (14).

The negligible influence of C:F ratio on total lipid in livers of rats fed CO or HCO with fructose indicates that with these diets, in contrast with those containing glucose, effects of carbohydrate-fat combinations predominated regardless of the proportions in which the 2 nutrients were fed. The apparent synergy of fructose and HCO in the HC:LF diet suggests that activity of the pathway(s) by which fructose is metabolized, at least when it is the major source of calories, enhances the mechanism(s) by which dietary HCO can induce fatty livers. Food efficiency data (lower values for F-HCO than for corresponding G-HCO groups) are consistent with accentuation of EFA deficiency by fructose. Also, the influence of fructose in lessening the degree of lipid accumulation in livers of rats fed at high levels of CO probably reflects different relationships between pathways of fructose and lipid metabolism on the one hand, and between glucose and lipid metabolism on the other.

Data from enzyme assays suggest that differences between responses to LC:HF diets as compared with HC:LF diets reflect differences in relative activities of alternate pathways, rather than simple reduction of throughput with diets containing less carbohydrate. The fact that G-6-Pase activity at 2 weeks was identical in the 2 control groups (G-CO) indicates that the C:F ratio per se cannot explain the striking differences in response to the 2 ratios of the other carbohydrate-fat combinations (fig. 2).

The stimulating effect of HCO on G-6-Pase activity, more pronounced with the higher fat diet, could be attributed either to increased glucose utilization associated with EFA deficiency or to metabolic effects of the fatty acids of HCO. The data support the latter possibility, in that stimulation of G-6-Pase activity by diets containing 5% of HCO with glucose was less at 4 weeks than at 2 weeks, but degree of EFA deficiency increases with time. Also, in a previous study (2), an EFA-deficient diet containing hydrogenated peanut oil did not stimulate G-6-Pase activity, suggesting that the effect of HCO on glucose metabolism was associated with utilization of the shorter chain fatty acids of HCO.

The above results show that the ratio of carbohydrate-to-fat in the diet can influence metabolic responses to different combinations of the 2 nutrients. The data further suggest that effects of altering the carbohydrate-to-fat ratio involve more fundamental adaptations than direct variation in throughput in proportion to absolute amounts of individual nutrients in the diet. Such interrelationships could have important implications relative to evaluating effects of specific dietary fats or carbohydrates.

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Utilization of Calcium and Sodium Sulfate by the Rat [1,2]

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ABSTRACT In order that calcium sulfate might be established as a satisfactory source of inorganic sulfur to use in altering the ratio of neutral to inorganic sulfur in purified diets, salt mixtures incorporating calcium sulfate and sodium sulfate at levels sufficient to give 0.10 and 0.42% of inorganic sulfur in the diet were compared with each other and with the Hubbell-Mendel-Wakeman salt mixture. The comparative effect of these salts on growth, feed efficiency, absorption, fecal and urinary excretion, blood level and the incorporation of sulfate into cartilage mucopolysaccharides was studied. Calcium sulfate appeared to function as well as, if not better than, sodium sulfate; no ill-effects of either salt were observed. When the animals received 0.10% and 0.42% of sulfate a small increase in feed efficiency was obtained over that recorded with 0.02% of sulfate as furnished by the level of Hubbell-Mendel-Wakeman salt mixture used in this investigation.

In planning experiments designed to investigate the interrelationships between vitamin E and sulfur metabolism it has been advantageous to alter the ratio of neutral to inorganic sulfur in the diets of rats.

High levels of inorganic sulfur in diets have most frequently been obtained by the addition of sodium sulfate (1–5). Calcium sulfate has been chosen for the salt mixtures used in this laboratory to facilitate changes in the level of sulfate in the diet without changing the sodium level. Consideration of the different chemical and physical properties of calcium and sodium sulfate indicated that a comparison of the utilization of these salts was desirable to establish that the results obtained with these salt mixtures were due to alterations of dietary sulfur and not to the choice of a toxic or unavailable salt.

It is the purpose of this paper to present data which compare the utilization of calcium and sodium sulfate by the rat. Since sulfation of cartilage mucopolysaccharides has been shown to have sufficient sensitivity for assay of growth hormone (6), growth, feed efficiency, absorption, fecal and urinary excretion, and blood level data have been supplemented with cartilage mucopolysaccharide sulfation data.

EXPERIMENTAL PROCEDURES

Littermate sets of albino (Wistar strain) rats were used throughout this investigation. Adult female rats were used in the first study, and weanling rats, in the second study. In the latter study animals were weaned and given their respective experimental diets at 21 days of age. The composition of the basic diet, a modification of the diets of Pendergrass,[6] is shown in table 1. The inorganic sulfur content was varied through modifications of the salt mixture of Hubbell et al. (7) as shown in table 2. Distilled water was made available to the rats at all times.

In the adult study, 5 sets of four littermate, adult, female animals from our stock colony were fed for 17 days diets containing salt mixtures to give 0.42 or 0.10% of sulfate in the diet as calcium sulfate or as sodium sulfate. The added methionine was eliminated from the diet shown in table 1 when the 0.42% salt mixture was used in order that the total sulfur, calculated as sulfate from neutral and inorganic sources, in the diet might remain constant.

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[2] Published by permission of the Director, Tennessee Agricultural Experiment Station.

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TABLE 1

Composition of the basic experimental diet

	g/100 g of diet
Casein (vitamin-free)	15.00
DL-Methionine	0.35
Sucrose	30.65
Cornstarch	32.00
Stripped lard [1]	6.00
Cod liver oil	2.00
Non-nutritive bulk [2]	10.00
Vitamin mixture [3]	1.00
Salt mixture [4]	3.00

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

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Each 100 g of diet were supplemented with the following synthetic vitamins: (in mg) nicotinic acid, 20.0; pyridoxine·HCl, 0.5; thiamine·HCl, 0.5; riboflavin, 0.5; Ca pantothenate, 1.0; folic acid, 0.5; biotin, 0.005; 2-methylnaphthoquinone, 0.025; vitamin B_{12}, 0.0045; choline chloride, 100.0; *i*-inositol, 100.0; *p*-aminobenzoic acid, 7.5. These vitamins were triturated in sucrose, q.s. to make 1.0 g. *dl*-α-Tocopheryl acetate was added to the diet at the level of 28 mg/100 g of diet.

[4] See table 2.

After the first 7 days a diet with these salts containing ^{35}S was substituted for the remainder of the experimental period. Total fecal collections were made during the 10-day period of the radioactive diet. Urine collection was made on the day before termination. Animals were decapitated following light ether anesthesia. Blood was collected in isotonic sodium citrate and intercostal cartilages were removed and stored at − 20°.

In the growth study, 6 sets of 3 male rats were raised from weaning to approximately 10 weeks of age with diets containing 0.42% of sulfate as calcium sulfate or as sodium sulfate and a diet containing 0.02% of sulfate from the mixture of Hubbell et al. (7). In this study the methionine supplementation was not omitted from the 0.42% of sulfate diets; hence their total sulfur was 0.99% compared with 0.49% for the 0.02% of sulfate diets. An additional 6 sets of 3 females were raised with diets containing 0.10% of sulfate as either of the 2 salts mentioned above and the control diet containing 0.02% of sulfate. Growth and feed intake records were kept. At the end of the growth period, the animals receiving the sodium and calcium sulfate salts were supplied with diets containing these salts in radioactive form for 10 days and then decapitated following light ether anesthesia. Total urine and fecal collections were made throughout the latter period. Blood and intercostal cartilage were collected at the time of killing.

Radioactive calcium sulfate was prepared by adding stoichiometric amounts of $H_2{}^{35}SO_4$[7] to a saturated solution of calcium lactate. The resulting precipitate was washed and dried. Radioactive sodium sulfate was prepared by neutralizing $H_2{}^{35}SO_4$ with NaOH, adding carrier Na_2SO_4 and drying the salt.

The specific activities of the diets, blood, feces and urine were determined by com-

[7] Obtained from Oak Ridge National Laboratories, Oak Ridge, Tennessee.

TABLE 2

Composition of the salt mixtures [1]

Substance	% Sulfate in the diet				
		as $CaSO_4$		as Na_2SO_4	
	0.0002	0.42	0.10	0.42 [2]	0.10
	g	*g*	*g*	*g*	*g*
$CaCO_3$	44.750	30.346	41.250	44.750	44.750
$MgCO_3$	3.060	3.060	3.060	3.060	3.060
$CaSO_4 \cdot 2H_2O$	—	25.097	6.000	—	—
$Na_2SO_4 \cdot H_2O$	—	—	—	20.720	4.943
$MnCl_2 \cdot 4H_2O$	0.040	0.040	0.040	0.040	0.040
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072	0.072	0.072	0.072	0.072
Cornstarch	10.693	—	8.193	—	5.750

[1] The salt mixture of Hubbell et al. (7), contained: (in g) $CaCO_3$, 54.300; $MgCO_3$, 2.500; NaCl, 6.900; KCl, 11.200; KH_2PO_4, 21.200; $FePO_4 \cdot 2H_2O$, 2.050; KI, 0.008; NaF, 0.010; $AlK(SO_4)_2$, 0.017; $MgSO_4 \cdot 7H_2O$, 1.600; $MnSO_4 \cdot H_2O$, 0.035; $CuSO_4 \cdot 5H_2O$, 0.090; and contributed 0.02% of sulfate when added to the diet at the level of 3%. It was modified to contribute appropriate levels of sulfate to the diets. The $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$ and $CuSO_4 \cdot 5H_2O$ were eliminated; the additions and adjustments in the $CaCO_3$ and $MgCO_3$ appear in the table.

[2] This salt mixture contained an additional 10 g of material. It was added to the diet (table 1) at the level of 3.3%, replacing a part of the cornstarch, in order that all other mineral constituents would remain constant.

TABLE 3

Distribution of radioactivity in the feces, blood, and urine and mucopolysaccharides of adult rats fed ^{35}S-containing sulfate salts [1]

Dietary sulfate level [2]	Feces	Blood	Urine	Mucopoly-saccharides
	%	%	%	%
0.42% as:				
$Ca^{35}SO_4$	6.15 ± 0.96	0.14 ± 0.010	1.68 ± 0.42	0.78 ± 0.04
$Na_2{}^{35}SO_4$	8.38 ± 1.86	0.19 ± 0.036	2.72 ± 0.58	0.50 ± 0.14
0.10% as:				
$Ca^{35}SO_4$	8.24 ± 1.68	0.15 ± 0.04	2.16 ± 0.24	0.56 ± 0.04
$Na_2{}^{35}SO_4$	10.26 ± 1.94	0.17 ± 0.016	3.26 ± 0.56	0.75 ± 0.08

[1] Results, count/min per mmole $SO_4^=$ as a percentage of total count/min ingested in the feed, are averages of 5 females ± the SE of the mean.
[2] See table 2.

busting the appropriate samples according to the method of Katz and Golden (8) and counting the precipitated $BaSO_4$ in an automatic gas-flow counter. The specific activity of the ester sulfate of the sulfomucopolysaccharides, isolated after the method of Bostrom (9), was determined by the method of Dodgson and Rice (10), adding sufficient carrier sulfate to give 12 mg of $BaSO_4$ and collecting and counting this salt as in the above. Creatinine determination was made by the method of Folin (11) on aliquot portions of the urine and the counts per minute were expressed per milligram of creatinine in the sample. The significance of the differences obtained was evaluated by the method of paired observations (12).

RESULTS

The data obtained for the specific activity of the feces, blood, urine and cartilage mucopolysaccharides from the study using adult female rats are presented in table 3. These data have been expressed as counts per minute per mmole of sulfate as a percentage of the total counts per minute ingested. This method of presenting the data was chosen since it eliminates errors due to the unavoidable variations in isotope ingestion associated with different feed consumption rates and the consumption of diets with different specific activities from period to period as shown in table 4. The average percentage of ingested radioactivity which was excreted in the feces tended to be lower from the calcium sulfate than from the sodium sulfate diets and from the diets with the 0.42% level of sulfate when compared

TABLE 4

Total feed intake and isotope consumption [1]

Dietary sulfate level [2]	Feed intake	
	g	*count/min* × 10^{-5}
0.42% as		
$Ca^{35}SO_4$	116 ± 3.4	15.5 ± 3.6
$Na_2{}^{35}SO_4$	111 ± 4.9	8.1 ± 2.9
0.10% as		
$Ca^{35}SO_4$	123 ± 10.0	19.4 ± 4.2
$Na_2{}^{35}SO_4$	119 ± 6.6	11.9 ± 1.4

[1] Averages of 5 females ± the SE of the mean.
[2] See table 2.

with those containing the 0.10% level. The sulfur compounds of the blood of the animals receiving the calcium sulfate diets had a lower percentage of the ingested $^{35}SO_4^=$ than did the animals receiving sodium sulfate. This difference between the 2 sulfate sources at the higher dietary sulfate levels was statistically significant ($P < 0.05$). The highest percentage of dose in mucopolysaccharide was noted in the animals receiving the higher level of $CaSO_4$, which had the lowest fecal and urinary excretion and blood level. This was significantly higher than that from the 0.10% $CaSO_4$ diet and higher than the mucopolysaccharides from 0.42% Na_2SO_4 diet. The 0.10% level of Na_2SO_4 resulted in a significantly higher percentage uptake into the mucopolysaccharides than the same level of $CaSO_4$.

Because these data were obtained with adult rats which had been fed the diet a relatively short time, 17 days, it seemed advisable to determine the effect of feeding diets incorporating these salt mixtures to rats during their growth period. The data which represent the weight gain and

feed efficiency ratios for rats raised with these diets for 10 weeks are presented in table 5. The growth of the animals receiving diets containing 0.42% of sulfate as compared with 0.02% of sulfate was not significantly different with either calcium or sodium sulfate as the sulfate source. The feed efficiency, calculated by dividing the grams of diet eaten into the grams of weight gained, showed the feed utilization of both male and female rats fed diets containing 0.42% of sulfate as $CaSO_4$ to be significantly better than that of those fed the 0.02% of sulfate diet ($P < 0.05$).

When the 0.10% levels of sulfate as either salt in the diet were compared with the 0.02% of sulfate diet, there was no significant difference in the growth of the animals. In the total 10-week period the 0.10% of $CaSO_4$ mixture gave significantly higher feed efficiency ratios than either the Hubbell, Mendel and Wakeman mixture or the Na_2SO_4 mixture incorporated into the diet of the female rats. (Littermates were used only among the 3 diets compared; beginning weight and growth sometimes varied from one litter to another.) Since these animals were not littermates, the 2 experimental sulfate levels were not compared statistically.

After these animals had been fed the diets approximately 12 weeks, those receiving 0.10 and 0.42% of sulfate as either calcium or sodium sulfate were given radioactive diets for 10 days. Table 6 shows the feed intake in terms of grams and of total counts per minute of the ^{35}S, as well as the total counts per minute for the excreted feces and the percentage of the total radioactivity absorbed. An attempt was made to keep the radioactivity of these diets similar, and the only statis-

TABLE 5

Average weight gain and feed efficiency ratios of rats raised with experimental diets for 10 weeks [1]

Level and source of sulfate in diet [2]	Weight gain		Feed efficiency ratio	
	♂	♀	♂	♀
	g	*g*		
0.42% ($CaSO_4$)	296±7	173±5	0.30±0.002	0.22±0.004
0.42% (Na_2SO_4)	316±6	177±5	0.29±0.004	0.23±0.001
0.02% (H–M–W)[3]	306±12	183±6	0.26±0.012	0.20±0.004
0.02% (H–M–W)	300±8	178±4	0.28±0.005	0.21±0.005
0.10% ($CaSO_4$)	304±5	188±5	0.28±0.004	0.23±0.002
0.10% (Na_2SO_4)	310±4	182±5	0.28±0.006	0.22±0.010

[1] Results are the average of 6 animals, with the exception of the 0.42% of sulfate female groups which are the averages of 5 animals, ± the SE of the mean.
[2] See table 2.
[3] Hubbell et al. (7).

TABLE 6

Radioactivity ingested in the feed by the animals in the growth study [1]

Level of sulfate in diet [2]	Feed intake	Radioactivity		
		Ingested	Fecal excretion	Absorbed
	g	*total count/min* × 10^{-5}		%
Males				
0.42% ($Ca^{35}SO_4$)	129±5	13.0±1.4	2.4±0.18	81±2
0.42% ($Na_2{}^{35}SO_4$)	121±10	9.8±1.2	2.5±0.35	74±2
0.10% ($Ca^{35}SO_4$)	143±6	18.0±2.0	4.3±0.36	73±5
0.10% ($Na_2{}^{35}SO_4$)	162±4	13.0±0.6	5.2±0.36	59±5
Females				
0.42% ($Ca^{35}SO_4$)	100±4	14.0±1.1	2.3±0.18	84±1
0.42% ($Na_2{}^{35}SO_4$)	98±8	24.0±1.3	2.2±0.20	89±2
0.10% ($Ca^{35}SO_4$)	97±8	18.0±5.7	3.7±0.82	72±7
0.10% ($Na_2{}^{35}SO_4$)	92±4	16.0±1.6	4.6±0.52	70±4

[1] See footnote 1 of table 5.
[2] See table 2.

TABLE 7
Distribution of radioactivity in feces, blood, urine and mucopolysaccharides in animals in the growth study [1]

Dietary sulfate level [2]	Feces [3]	Blood [4]	Urine [5]	Mucopoly-saccharides [6]
	%	%	%	%
Males				
0.42% ($Ca^{35}SO_4$)	19 ± 2.4	0.046 ± 0.015	1.2 ± 0.10	0.87 ± 0.13
0.42% ($Na_2{}^{35}SO_4$)	26 ± 2.1	0.012 ± 0.002	1.8 ± 0.12	0.88 ± 0.26
0.10% ($Ca^{35}SO_4$)	27 ± 4.7	0.018 ± 0.002	1.1 ± 0.22	1.55 ± 0.22
0.10% ($Na_2{}^{35}SO_4$)	41 ± 4.7	0.030 ± 0.003	2.6 ± 0.95	1.80 ± 0.46
Females				
0.42% ($Ca^{35}SO_4$)	16 ± 1.2	0.010 ± 0.001	1.3 ± 0.18	0.37 ± 0.10
0.42% ($Na_2{}^{35}SO_4$)	11 ± 2.5	0.009 ± 0.004	1.5 ± 0.38	0.27 ± 0.10
0.10% ($Ca^{35}SO_4$)	28 ± 7.0	0.017 ± 0.004	0.76 ± 0.30	0.54 ± 0.20
0.10% ($Na_2{}^{35}SO_4$)	30 ± 4.1	0.016 ± 0.002	1.4 ± 0.50	0.46 ± 0.20

[1] See footnote 1 of table 5.
[2] See table 2.
[3] Total count/min, expressed as a percentage of the total ingested radioactivity.
[4] Count/min per ml of whole blood, expressed as a percentage of the total ingested radioactivity.
[5] Count/min per mg of creatinine, expressed as a percentage of the total ingested radioactivity.
[6] Count/min per mmole of $SO_4^=$, expressed as a percentage of the total ingested radioactivity.

tically significant difference in the counts per minute consumed was between the groups of rats fed the diets containing 2 forms of sulfate at 0.42%. The female rats fed the calcium sulfate consumed less ^{35}S ($P < 0.05$), and the male rats fed the sodium sulfate consumed the least ($P < 0.05$). There was a significantly lower percentage excretion of the radioactivity from the female rats fed the diet containing 0.10% of sulfate as $CaSO_4$ than from those fed the diet containing 0.10% of sulfate as Na_2SO_4. A higher percentage sulfate absorption was obtained with those diets containing the largest amounts of inorganic sulfur.

The data in table 7 showing the distribution of the radioactivity in the material analyzed are presented as a percentage of ingested radioactivity. On the basis of the radioactivity of these tissues, there are only 2 statistically significant differences between the utilization of the sulfur of calcium and sodium sulfate by the growing rat. The male rats receiving the 0.42% of sulfate as calcium sulfate excreted significantly ($P < 0.05$) smaller quantities of ^{35}S in their urine than did those fed the 0.42% of sulfate as Na_2SO_4 diet. None of the other urinary excretions were significantly different. The blood of the male rats receiving 0.10% of sulfate as sodium sulfate contained significantly more radioactivity than that from those fed calcium sulfate. Analysis of variance (12) indicated that a significantly higher percentage of the ingested radioactivity was retained by the cartilage mucopolysaccharides of the male rats than by that of the female rats.

DISCUSSION

The results obtained using adult female rats showed that the inorganic sulfur from both calcium and sodium sulfate was absorbed and incorporated into tissues. The calcium salt was generally absorbed better than its sodium counterpart, and in all cases, in both the adult and growing studies, there was less of the sulfate from the calcium sulfate excreted in the urine. The total sulfur as sulfate in the diet from neutral plus inorganic sources was kept constant at 0.67%. As a result, both the inorganic and organic sulfate level of these diets were varied in an inverse manner. The animals receiving the higher levels of sulfate did not receive supplemental methionine; therefore, they received only 0.25% of the diet as sulfate from the organic sulfur sources in casein, whereas those fed the 0.10% of sulfate diets had 0.57% of organic sulfur. This could account for the higher absorption of $^{35}SO_4^=$ from the 0.42% of sulfate diets compared with their 0.10% counterparts. It appears that when the animal has less neutral sulfur to convert to sulfate it compensates by an increased absorption of inorganic sulfur.

The growth and feed utilization study with weanling rats revealed no ill-effects

from either of the salts at the levels used after long-term consumption. The growth appeared normal; the animals receiving the Hubbell, Mendel and Wakeman salt mixture gained about the same amount as stock animals in our laboratory. The differences in total dietary sulfur described previously may have been reflected in the increased feed efficiency which was obtained with the rats consuming the higher level of sulfate (0.42%) diets. If the lower level of inorganic sulfur (0.02%) is not sufficient to supply the sulfate needs of the rat, it may be forced to oxidize a large percentage of its neutral sulfur to satisfy its sulfate needs. As a result the sulfur amino acids could become limiting with a concomitant decrease in feed efficiency.

A statistically significant increase in the specific activity of the sulfur of cartilage mucopolysaccharides, expressed as a percentage of the dose, was obtained with the female rats fed 0.10% of sulfate as calcium sulfate (table 7). A higher specific activity in the mucopolysaccharides from animals receiving calcium sulfate diets compared with sodium sulfate diets was obtained, with one exception, in the adult study (table 3).

Because the adequacy of the basic salt mixture modified for use in these diets has been questioned (13), the effect of the addition of zinc and adjustment of the calcium-to-phosphorous ratio to agree with the NRC recommendations (14) was checked with weanling rats from our stock colony. No significant improvement in the growth rate was obtained when salt mixtures incorporating these additions were compared with the basic salt mixture used in these studies.

In view of the similarity of sulfur and seleno-compounds which generally results in contamination of sulfur compounds with selenium, it seemed desirable to check the effect of selenium addition to our low sulfate diets to be sure that the effects which we have observed were due to low sulfate and not to a selenium deficiency. Since pyruvic acid oxidase was one enzyme observed by Yang et al.[8] to be subject to selenium deficiency, the pyruvic acid oxidase activity of liver homogenates from rats fed the low sulfate and low sulfate with added selenium were checked. No significant difference was obtained. Therefore, it appears likely that these effects are due to diets low in sulfur.

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A Comparison of the Utilization of Organic and Inorganic Sulfur by the Rat [1,2]

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ABSTRACT This investigation compared the utilization of neutral and inorganic sulfur for the sulfation of cartilage mucopolysaccharides. Adult, female rats were fed experimental diets containing inorganic sulfur at 3 levels: 0.0002, 0.10 and 0.42% and total sulfur at constant levels for 17 days. During the last 10 days radioactive sulfur as sulfate or methionine was introduced into the diet and the incorporation into feces, blood and cartilage mucopolysaccharides was determined. The data presented show that with a normal diet inorganic sulfur contributes as much to the metabolic sulfate pool as does neutral sulfur. The data also indicate that the availability of $SO_4^=$ is related to the ratio of neutral to inorganic sulfur in the diet and that the intestinal absorption of methionine may be related to the inorganic sulfur level of the diet. This evidence suggested that there is a need for inorganic sulfur in the diet to prevent an increase in the sulfur amino acid requirement above that normally stated.

Work from many laboratories which has demonstrated a relationship between the sulfur-containing amino acids and vitamin E has been reviewed by Scott (1). Previous work in this laboratory has shown that the response of rats to avitaminosis E was related not only to the total sulfur in the diet, but also to the ratio of neutral to inorganic sulfur.[4] When rats were forced to satisfy their sulfate requirements by oxidation of neutral sulfur the effects of vitamin E deficiency appeared to be enhanced. The available literature (2, 3) places little importance upon the role of inorganic sulfur in mammalian nutrition and implies that inorganic sulfur in the diet is poorly utilized by non-ruminant mammals. Our observations seemed contradictory to the literature reports since, if inorganic sulfur were not a factor in the nutrition of the rat, the effect of avitaminosis E should be independent of the level of dietary sulfate.

Further studies in this laboratory (4) showed that rats absorbed a major portion of radioactive sulfate from dietary calcium or sodium sulfate and used it to sulfate cartilage mucopolysaccharides. The addition of sulfate improved the feed efficiency of animals fed diets that were adequate otherwise. There was some indication that the ratio of organic to inorganic sulfur played a role.

The present investigation was designed to compare the extent to which inorganic sulfur and neutral sulfur could be used for sulfation of natural products.

EXPERIMENTAL PROCEDURE

This study comprised 5 experimental periods and used a total of 20 rats. For each experimental period 4 adult female albino rats of the Wistar strain were fed one of the four diets shown in table 1 so that each littermate would consume a different test diet for 17 days. For the first week of the experimental period the diet was non-radioactive, during the last 10 days of the period the animals received the radioactive diets as shown in table 2. Feed and distilled water were made available to the rats at all times. Feed intake measurements and fecal collections were made daily while the animals were receiving the radioactive diets. At the end of the period the animals were decapitated and their rib cartilage and blood collected. The deter-

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[2] Published by permission of the Director, Tennessee Agricultural Experiment Station.

[3] Holder of a General Foods Fellowship during this study.

[4] Pendergrass, B. J. 1961 The interrelationship of tocopherol and sulfur metabolism. Unpublished Master's Thesis, University of Tennessee.

TABLE 1

Composition of the diets

	A	B	C	D
	g/100 g of diet			
Casein	15.00	15.00	15.00	15.00
DL-Methionine	0.00	0.35	0.35	0.60
Sucrose	31.00	30.65	30.65	30.40
Cornstarch	32.00	32.00	32.00	32.00
Non-nutritive bulk [1]	10.00	10.00	10.00	10.00
Lard	6.00	6.00	6.00	6.00
Cod liver oil	2.00	2.00	2.00	2.00
Vitamin mixture [2]	1.00	1.00	1.00	1.00
Salt mixture [3] with 0.42% $CaSO_4$	3.00	—	—	—
Salt mixture with 0.10% $CaSO_4$	—	3.00	3.00	—
Salt mixture with 0.0002%	—	—	—	3.00

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] Described in the first of this series (4) in table 1, footnote 2.
[3] Described in the first of this series (4) in table 2.

TABLE 2

Calculated levels of dietary sulfur and distribution of radioactivity

Diet	% Sulfur as sulfate		Total
	Inorganic	Neutral	
			%
A	0.42 ($Ca^{35}SO_4 \cdot 2H_2O$) [1]	0.25	0.67
B	0.10	0.57 (^{35}S-methionine) [2]	0.67
C	0.10 ($Ca^{35}SO_4 \cdot 2H_2O$)	0.57	0.67
D	0.00	0.67 (^{35}S-methionine) [2]	0.67

[1] Prepared as described previously (4).
[2] Purchased from Schwartz Bioresearch Inc., Mount Vernon, New York. The purity was confirmed by paper chromatography.

mination of the specific activities of the diets, blood, feces and mucopolysaccharides has been described (4). The significance of the differences obtained was evaluated by the method of paired observations (5).

RESULTS

The data which are presented in table 3 show that there was no statistically significant difference in the feed consumption of the rats fed any of the diets, and perhaps because of the large standard error of the mean, there was no statistically significant difference in the total isotope consumption.

Data shown in table 4 represent the specific activity of the feces, blood and cartilage mucopolysaccharides. For reasons presented previously (4) these data are expressed as the specific activity of sulfate as a percentage of the ingested radioactivity. The only significant difference in the fecal excretion is between diets C and D ($P < 0.005$) which may indicate a preferential absorption of methionine. There

TABLE 3

Total feed intake and isotope consumption [1]

Diet	Feed intake	Feed intake
	g	*count/min* $\times 10^{-5}$
A	116± 3.4	15.5±3.6
B	115± 2.6	10.2±5.3
C	123±10.0	19.4±4.2
D	116± 5.1	12.2±3.7

[1] Data are averages of 5 animals ± SE of mean.

TABLE 4

Distribution of radioactivity in the feces, blood and mucopolysaccharides [1]

Diet	Feces	Blood	Mucopoly-saccharides
A	6.15±0.96	0.14±0.009	0.78±0.043
B	6.35±1.86	4.09±0.60	0.64±0.098
C	8.24±1.68	0.15±0.004	0.56±0.035
D	5.06±0.99	2.73±0.41	0.39±0.042

[1] Data are the averages of 5 trials, expressed as count/min per mmoles $SO_4^=$ as a percentage of total ingested radioactivity ± SE of the mean.

is a highly significant difference ($P < 0.001$) in the specific activity of the blood from all animals except those that consumed the radioactivity as $Ca^{35}SO_4$, diets A

and C. The specific activity of rib cartilage mucopolysaccharides obtained from the rats fed the high inorganic sulfur diet, diet A, is higher than that obtained from rats fed any of the other diets and significantly higher than that from rats fed the 0.10% of sulfate diet, diet C, ($P < 0.005$) or those where most of the sulfate had to be produced by oxidation of organic sulfur, diet D, ($P < 0.02$). The specific activity of the cartilage mucopolysaccharides from the rats fed the radioactive methionine-supplemented diet containing 0.10% of inorganic sulfate, diet B, was significantly higher than the mucopolysaccharides from those fed the radioactive methionine-supplemented diet which did not contain any inorganic sulfur, diet D, ($P < 0.03$).

DISCUSSION

Denko and Bergenstal (6) have suggested that fixation of ^{35}S by cartilage mucopolysaccharides may have sufficient sensitivity to be used in the assay of growth hormone. Therefore, it appears reasonable to assume that the data which have been presented for the sulfation of cartilage mucopolysaccharides have shown that with diets which contain at least 0.10% of inorganic sulfate rats use both inorganic and organic sulfur equally well for sulfation of mucopolysaccharides and that the level of inorganic sulfur in the diet may have an effect on the neutral sulfur requirement of the rat. It is conceivable that the inorganic sulfate which was fed may have been reduced either by the intestinal flora or as shown by Robinson (7) by the intestinal mucosa; however, the data obtained for the specific activity of the blood demonstrate that there was little likelihood of the ingested inorganic sulfur existing to any appreciable extent as organic sulfur in the blood. For example, the specific activity of the sulfur was very low in the blood from those rats that obtained the radiation from inorganic sulfur compared with the 20 to 40 times increase in specific activity in the blood of those rats consuming diets with the label in the organic sulfur. Since it is evident that a high specific activity of the sulfur in the blood is associated with the organic forms, it does not appear likely that these differences would exist if significant quantities of inorganic sulfur were reduced by the intestinal flora. Waldschmidt (8) has suggested that very little sulfate was reduced by the rat.

The sulfate incorporation of the mucopolysaccharides from rats fed the diet containing radioactive methionine and 0.10% of inorganic sulfur, diet B, was significantly higher than that from rats fed the radioactive methionine diet which contained no inorganic sulfur. Interpretation of these data is complicated by the fact that in order to keep the total "sulfate" content of the diets constant, inorganic and neutral sulfur were varied in an inverse manner. Therefore, there are more sulfur-containing amino acids in the diet without inorganic sulfur which would tend to dilute their specific activity. However, in the other diet the specific activity of the sulfate formed from neutral sulfur would be diluted by the unlabeled inorganic sulfur which has been shown to be utilizable. If the contribution of the inorganic sulfur is ignored, there is still an apparent decrease in sulfate incorporation by the mucopolysaccharides from rats fed the diet without inorganic sulfur.

The ratio of sulfur amino acids in diet D to B, assuming complete digestibility of the casein, is 1.10 to 0.85 g/100 g of diet. Diet D has 0.60 g plus 0.50 g from the casein; diet B, 0.35 g plus 0.50 g from the casein; diet B has only 77% of the sulfur amino acids contained in diet D. If the percentage specific activity, 0.64, obtained for the mucopolysaccharides from the rats fed diet B is multiplied by 77%, 0.49 is obtained, a figure which is still higher than the 0.39 obtained from the animals fed diet D. These data are strengthened by the lower specific activity of the blood and feces of the rats fed diet D compared with diet B and indicate that 1.1% of sulfur amino acids in the diet which contains no inorganic sulfur may be inadequate, whereas 0.6% as reported by Womack et al. (9) is adequate if the diet contains inorganic sulfur. At this time we have no indication of the minimal amount of inorganic sulfur necessary; however, it appears that 0.1% is adequate and probably somewhat less will suffice (4).

An exhaustive search of the literature did not reveal a specific dietary requirement for inorganic sulfur by any mam-

malian species. In general it is stated that the inorganic sulfur requirement of the animals can be met by in vivo oxidation of neutral sulfur. Wellers et al. (10) reported that sulfate sulfur could supply about one-third of the total sulfur requirement and all of the sulfur required beyond that of amino acids. So far as the authors are aware this is the first indication of a need for inorganic sulfur in the diet if the sulfur amino acid requirement is not to be increased.

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Effect of Environmental Temperature on Utilization of Dietary Protein by the Growing Rat

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ABSTRACT It has been shown that, provided the energy intakes of animals are below a certain physiological maximum, the thermogenesis resulting from cold exposure is accompanied by an increased food consumption, and an improved growth and N balance as compared with animals at thermal neutrality. Under these conditions, efficiency of protein utilization, rate of total endogenous N loss, and the energy cost of protein synthesis were shown to be unchanged. When the temperature, and dietary regimen were such that the animal's maximum food intake was inadequate to meet its energy needs for growth and thermogenesis, the efficiency of protein utilization was reduced as compared with that at normal temperatures, in a manner analogous to the reduction observed at restricted caloric intakes. Equations are presented, for prediction of effective protein values of diets under conditions of caloric restriction and reduced temperature. The significance of these observations is discussed in relation to the optimal conditions for efficient feeding of livestock, and also the standardization of conditions under which measurements of protein quality should be made.

There have been many experiments which demonstrate that exposure of animans to cold results in increased food intake, accompanied by an elevated urinary loss of N, evidently related to the concomitant increase in protein-intake. Kleiber and Dougherty (1) showed that when chicks were fed a diet of constant composition at decreasing environmental temperatures, the intake of metabolizable energy rose progressively from 200 kcal/day/(kg body wt)$^{0.75}$ at 40°, to 300 kcal/day/(kg body wt)$^{0.75}$ at 21°, the net energy having a maximum between 30 and 40°, and growth rate being maximal at 21°. Exposure of rats to lower temperatures has been observed by most workers to exert an adverse effect on growth rates. For example, Sellers et al. (2) maintained rats at 1.5° with diets of various composition, the caloric density of which ranged from 4.1 to 6.2 kcal/g. Only very gradual weight increases were observed, and the caloric intakes which were the same for all the diets, were around 300 kcal/day/kg$^{0.75}$. At this temperature, growth and survival were unaffected by level of protein, fat or carbohydrate. However, experiments by other workers have indicated that exposure to cold may exert an advantageous effect at some levels of dietary protein, but not at others. Thus Tredwell et al. (3) showed higher growth rates and protein efficiency ratios at 1° than at 25° for diets having 5 and 10% protein, but poorer performance for diets having greater than 10%. Similarly Meyer and Hargus (4) noted greater growth rates at 2° than at 25° for rats fed a 10% casein diet but poorer performance at 2° than at 25° for rats fed a diet containing 25% casein. The data presented by these authors also indicate food intakes at low temperatures of a little over 300 kcal/day/kg$^{0.73}$.

The results presented in this paper, are of experiments designed to test the hypothesis that these effects can be explained on the following assumptions.

1) That protein metabolism *per se* is uninfluenced by environmental temperature. Specifically that, provided energy supply is adequate, the efficiency of utilization of dietary proteins, the energy cost of protein anabolism and total minimum endogenous N losses are independent of temperature.

2) There is a maximum rate of utilization of dietary energy (of approximately 300 kcal/day/kg$^{0.73}$ in the rat) which cannot be exceeded at any temperature, and which is independent of the caloric density of the diet.

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The first assumption suggests that the net protein utilization (NPU) and net dietary protein calories per cent (NDpCal%) of diets will not vary with temperature, so long as energy requirements for basal needs, temperature maintenance, and the energy cost of growth, are all satisfied. Hence it would be expected that increased intake of utilizable protein leading to increased N balance and weight gain, would result from the appetite response to reduced temperatures. However, the second assumption suggests that for each fixed temperature there will be a maximum possible rate of protein anabolism, and hence a maximum NDpCal% value which can be supported. Diets having values greater than this (as measured under normal, i.e., thermoneutral conditions), would give an impaired utilization analogous to that observed under conditions of caloric restriction.

Miller and Payne (5) demonstrated that for the range of caloric intakes within which energy supply is the factor limiting protein anabolism, the NDpCal% of diets can be predicted from the equation:

$$\text{NDpCal\%} = P\theta = \frac{100}{E}\left(1 - \frac{C_B}{C}\right) \qquad (1),$$

Where

θ = net protein utilization,
P = protein calories per cent of the diet,
E = kilocalories required for the synthesis of 1 kcal of protein,
C_B = kilocalories required for basal metabolism, and
C = caloric intake in kilocalories.

If during exposure to a temperature T, below the range of thermal neutrality, the metabolic rate is increased to a value C_T, equation 1 becomes:

$$P\theta = \frac{100}{E}\left(1 - \frac{C_T}{C}\right) \qquad (2).$$

Also if C_M = the maximum possible intake of metabolizable energy (assumption 2), the maximum possible NDpCal% at temperature T, will be:

$$\frac{100}{E}\left(1 - \frac{C_T}{C_M}\right) \qquad (4).$$

Diets having values for NDpCal% less than this, would be expected to promote increasingly higher growth rates and N balances with reduction of temperature down to T, and progressively poorer performance for temperatures below T. All diets having values greater than that given by equation 4, when measured at normal temperatures, would be expected to promote the same growth rates and N balances at temperatures below T, irrespective of their composition.

METHODS

Thirty-day-old rats were exposed to various environmental temperatures in still air by mounting the cages inside a refrigerator, having an air temperature regulator giving a control precision of ± 1°. No bedding material was provided, but the rats were housed in groups of four. The rats were not acclimatized previously.

The utilization of dietary protein was measured as net protein utilization operative, NPU by the method of Miller and Bender (6) as modified by Miller and Payne (7), body nitrogen content being determined by direct analysis. Calorific values of carcasses were determined by the method of Miller and Payne (8). Body fat content was calculated from the protein and gross energy content of dried carcasses.[1] Diets were constructed according to the formula given by Miller and Bender (6), casein being substituted for maize starch to give levels of 4, 10 and 25% protein. N balances were calculated using the equation given by Miller and Payne (5).

$$\Delta B = \frac{P\theta C}{2.5} - 250 \qquad (5)$$

where ΔB = N balance mg N/day/$kg^{0.73}$.

RESULTS

Choice of temperature. It was considered desirable for the main experiments, to choose an environmental temperature which would induce less than the maximal food intake, at least for some of the protein levels. Table 1 shows the results of a preliminary experiment to determine food intakes using 4 and 10% protein levels, at 3 environmental temperatures.

At 15° the food intakes with both diets were considerably higher than at 27° (the normal animal room temperature), but below those observed at 10°. Accordingly

[1] Jacob, M. 1963 The relationship between energy expenditure and protein in the dietary regimen. MSc Thesis. University of London.

TABLE 1

Caloric intakes of rats exposed to different air temperatures

Protein content of diet	Food intakes at environmental temperatures of		
	27°	15°	10°
%	*kcal/day/kg$^{0.73}$*		
4	150	220	290
10	210	290	304

15° was selected as a suitable temperature for the main experiments.

Net protein utilization at 27° and 15°. Diets containing zero, 4, 10 and 25% casein, were fed to groups of four 30-day-old rats housed at 27° and 15°. At the end of 10 days, the animals were killed, and the bodies analyzed for N and fat. Table 2 shows mean values for caloric intakes for all the diets and temperatures; these data indicate that exposure to 15° stimulated food intakes which exceeded those at 27° by approximately the same amount at all protein levels. A typical set of growth curves is shown in figure 1, which illustrates that rats fed a 4% casein diet grew slightly better, and those fed 10% casein diets markedly better at 15° than those fed the same diet at 27°. In contrast with this, rats fed the 25% diet grew more slowly at the lower temperature. Weight loss with the protein-free diet was the same at both temperatures.

Table 2 shows average values for weight changes, body composition, NPU, NDpCal% and N balance for all diets and temperatures, over the 10-day test period.

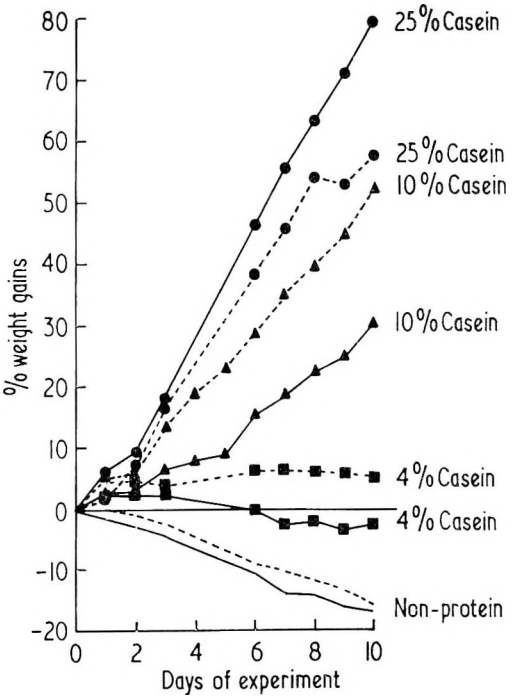

Fig. 1 Weight curves of rats fed diets containing casein at various levels, and housed at either 15 or 27°. . . . . 15°; ———— 27°.

There was no significant effect of temperature on the rate of total endogenous N loss, since the mean difference between the total body N content of the groups of rats after 10 days of consuming N-free diets at the 2 temperatures was only 0.6 ± 0.8% for 8 replicates. Table 2 shows that at the 4 and 10% protein levels, rats exposed to 15° ate more food, and utilized

TABLE 2

Utilization of N by rats fed diets of different protein content, at two temperatures

Diet	Temperature	Caloric intake	Body composition		NPU [1]	NDpCal%	Wt change	N balance
			N	Fat				
		kcal/day/kg$^{0.73}$	%	%			*g/day*	*mg/day/kg$^{0.73}$*
N-free	27°	124	9.6	26.7	—	0	−1.0	—
N-free	15°	188	10.0	23.0	—	0	−1.0	—
4% Casein	27°	160	9.2	30.0	68 ± 0 [2] (2) [3]	2.9	−0.1	−75
4% Casein	15°	222	9.2	30.0	69 ± 0.5 (2)	2.0	+0.2	+8
10% Casein	27°	226	8.8	35.0	66 ± 1 (3)	6.8	+1.8	+367
10% Casein	15°	316	9.3	31.0	64 ± 2 (3)	6.6	+2.6	+585
25% Casein	27°	240	9.9	28.0	55 ± 1 (2)	13.4	+4.1	+1030
25% Casein	15°	310	10.5	22.0	27 ± 1 (2)	9.0	+3.2	+870

[1] The differences between NPU's at the 2 temperatures are not significant for the 4 or 10% casein diets but are highly significant for the 25% casein diet.
[2] SE of mean.
[3] Number of replicate groups.

the concomitant extra protein with unimpaired efficiency, thus gaining more weight and nitrogen. However, animals fed the 25% protein diet were unable to utilize their dietary protein at full efficiency, and gained less weight and N at 15° than at 27°. The effect of temperature on carcass composition was slight, although with the exception of the groups fed 4% casein, the N contents tended to be higher, and fat contents lower at 15° than at 27°.

Effect of restricted caloric intake on NDpCal% measured at 15°. Equation 2 predicts that the relation between NDpCal % and $\frac{1}{C}$ will be linear, with an intercept of $\frac{100}{E}$ on the NDpCal% axis and $\frac{1}{C_T}$ on the $\frac{1}{C}$ axis. To test the validity of this, rats housed at 15° were fed the 25% casein diet in quantities corresponding to a range of from 160 to 310 kcal/day/kg$^{0.73}$. Figure 2 shows the values of NDpCal% obtained plotted against $\frac{1000}{C}$ together with the line fitted to the data by the method of least squares. Also shown is the line corresponding to previously reported values (9), which were obtained by feeding a number of different diets under conditions of thermal neutrality. The 2 lines, correlation coefficients − 0.96, (n = 16), and − 0.92 (n = 17) have intercepts corresponding to 6.2 (27°) and 6.7 (15°) kcal of metabolizable energy required for the anabolism of 1 kcal of protein (*E*). The intercepts on the calorie axis correspond to values of 70 kcal/day/kg$^{0.73}$ at 27°, which is the interspecies value for basal metabolism (10) and 130 kcal/day/kg$^{0.73}$ for C_T at $T = 15°$. Herrington (11) gives a formula for the metabolic rates of rats at various environmental temperatures, which predicts a value of 136 kcal/day/kg$^{0.73}$, for C_T.

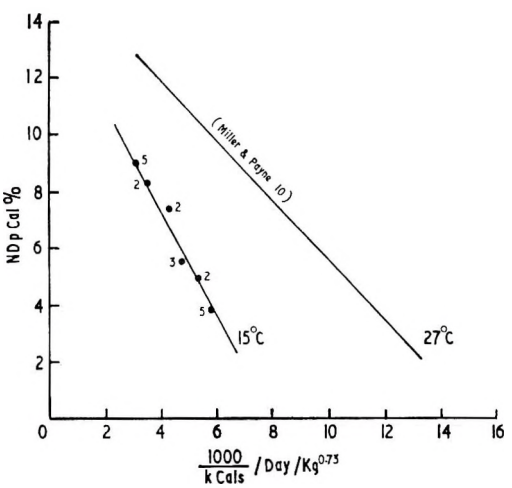

Fig. 2 Relationship between NDpCal% and caloric intake, measurements made at 15° (this paper) and 27° (Miller and Payne (9)). The experimental points shown are mean values, and the numbers indicate numbers of replicates.

DISCUSSION

In the introduction to this paper, 2 hypotheses were proposed, which if correct would explain some of the complex and in some ways conflicting evidence in the literature of the effect of environmental temperature on growth rate and food utilization.

The first hypothesis, namely, that protein metabolism as such is not qualitatively affected by a reduction in temperature, is supported by the evidence in the experimental section. Thus the NPU's of casein at concentrations of 4 and 10% were the same when fed at 15° as at 27° despite a large increase in protein intake. The fact that during all the experiments, the same amount of N was lost from the bodies of rats fed N-free diets at the 2 temperatures, shows that total endogenous N losses are unaffected by temperature reduction. Finally there is very good agreement between the value for *E*, the energy cost of protein anabolism measured at 15°, with the value previously determined under thermoneutral conditions.

The second hypothesis suggests that there is a fixed maximum possible rate at which energy can be metabolized by animals. This has been recognized for some time in relation to maximal feed intakes of rapidly growing animals, for example Kleiber (12) showed that for several species of animal ranging in size from chicks to steers, the ratio of the calorie value of the maximum food intake to basal metabolic rate is between 4 and 5. In the experiments reported here, no intakes greater than 310 kcal/day/kg$^{0.73}$ (about 4.5 times basal) were measured at any temperature. That this is not simply an

effect of bulk of food has been amply demonstrated, for example by Sellers et al. (2).

The observation that N metabolism is not qualitatively affected during thermogenesis is in accord with results of respiration studies of rat liver homogenates, which have shown that many of the Krebs cycle substrates including amino acids are oxidized at increased rates by the tissues of cold acclimatized animlas (13). If, as suggested by Miller and Payne (14), the rates of oxidation of amino acids and glucose are dependent upon their relative concentrations at the cellular level, an increase in overall oxidative capacity of the tissues would be accompanied by an increase in the amounts of substrates consumed, but there would be no change in their relative proportions. This is illustrated by the fact that under conditions of adequate energy intake and normal temperature, NPU is a function of concentration of protein, but not of intake (7, 9), and the present work shows that at the 4 and 10% level in the diet the increased amounts of protein eaten at 15°, were metabolized with unchanged efficiency.

Thermogenesis would result from the increased energy available from the above processes passing either directly to heat (a so-called uncoupled reaction), or indirectly through the formation of ATP, and the subsequent use of this for synthetic purposes. Heat developed indirectly through inefficient conversion of free energy along the latter route, would normally manifest itself as part of the heat increment of feeding, or specific dynamic action (SDA), and it is of interest to note, that the results of this paper indicate that in young growing rats, the heat produced as a result of the SDA of the diet, did not exert a compensatory effect on the amount of extra heat produced in response to cold; thus the experiments carried out using restricted food intakes in the cold resulted in a wide range of intakes and growth rates (and thus of SDA). However, the protein values measured were all consistent with equation 2, indicating that the increment in basal heat production was a constant. This observation, while the opposite of that of Rubner (15) using dogs, is in accord with results observed in pigs (16), and rabbits (17), and rats (18).

Application of the results of this work to other animals would be possible, given some estimate of maximal energy intake C_M, and of metabolic rate at the reduced temperature, C_T.

Kleiber (12) has suggested that the ratio of maximum food intake to basal metabolic rate is a constant for all species, and lies between 4 and 5. The value of ~ 300 kcal/day/kg$^{0.73}$ observed for rats by us, and by others, corresponds to 4.3 times the basal level.

Fasting metabolic rates at reduced temperatures can be predicted if the critical temperature is known, from the expression:

$$\frac{C_T}{C_B} = \frac{T_B - T}{T_B - T_C} \qquad \text{when } T < T_C$$

where T_B is the core or deep body temperature, and T_C the critical temperature below which the thermogenic response to cold is initiated. Using the values 39° and 27° for T_B and T_C for the rats, and taking C_B = 70 kcal/day/kg$^{0.73}$, the above expression gives C_T = 140 kcal/day/kg$^{0.73}$ at 15°, in reasonable agreement with the value derived indirectly in this paper.

Using the value of 17 for $\frac{100}{E}$ (Miller and Payne (9)), equation 4 thus becomes:

$$\text{NDpCal\%} = 17\left[\left(1 - \frac{70}{C}\right)\left(\frac{T_B - T}{T_B - T_C}\right)\right] \quad (6)$$

and inserting the value $C = 300$

$$= 17\left[(1 - 0.23)\left(\frac{T_B - T}{T_B - T_C}\right)\right] \quad (7)$$

Taking $T_B = 39°$, and $T_C = 27°$, values which according to Irving (19) are characteristic of tropical animals such as rats and nude men, we have:

$$\text{NDpCal\%} = 17\,[(1 - 0.019)\,(39 - T)] \quad (8)$$

for values of $T < 27°$.

A proper understanding of the relation between environmental temperature and efficiency of food utilization is of great practical importance for the most efficient housing of livestock, and for specifying the conditions under which biological assays for protein quality are to be carried out. Using equations 5 and 8, the temperature can be calculated at which maximum performance in terms of N balance will result from feeding a diet of known NDpCal% and also, for a given temperature, the

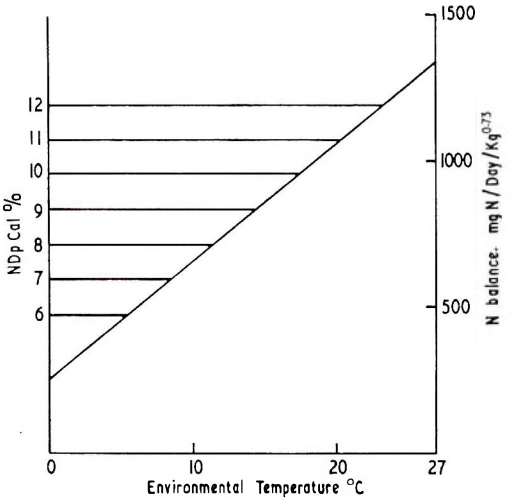

Fig. 3 Relationship between maximum possible N balance, and environmental temperature for diets of different NDpCal%. Assuming a maximum possible energy intake of 300 kcal/day/$kg^{0.73}$, and a critical temperature of 27°.

maximum value of NDpCal% in the diet which could be utilized. These points are illustrated in the diagram figure 3. The horizontal lines corresponding to different values of NDpCal% in the diet, intersect the slanting line at points indicating the minimum temperatures at which these values could be utilized, and the maximum possible N balance figures may be read from the right hand vertical axis. Thus, for example, a diet having an NDpCal% of 8.0 could bring about a maximum N balance of 710 mg/day/$kg^{0.73}$, at a temperature of 11.5°. Similarly values for NDpCal% greater than 11.0 could not be measured at environmental temperatures less than 20°.

Equation 6 may be used to predict the effects of restricted caloric intakes at different temperatures, and may be used to test the validity of the results of pair-feeding experiments, or of measurements of the protein value of diets fed in restricted quantities. Thus, for example, a diet having an NDpCal% of 8.0, when fed ad libitum to rats housed at 27°, would have a value of only 5.0 when fed in amounts equivalent to 100 kcal/day/$kg^{0.73}$. However, equation 6 indicates that the value would fall to zero if the latter measurement was made at a temperature of 22° (72°F).

Thus, an important condition for acceptable measurements of protein quality, and protein values of diets, is that these should be measured in the region of thermal neutrality, i.e., at a temperature of around 27° (81°F) for rats.

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Changes in Collagen Metabolism Caused by Feeding Diets Low in Inorganic Sulfur [1]

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ABSTRACT The effects of decreasing the level of dietary inorganic sulfur on the metabolism of skin collagen were studied. Significantly less neutral salt-soluble and total collagen was produced in the skins of rats fed diets which contained 0.0002% of sulfate compared with those fed diets containing 0.02% of sulfate. The soluble collagen solutions derived from skins of animals fed at the low level of sulfate would not form thermal gels which are typical of normal soluble collagen solutions. This information is indicative of a severe failure to form intramolecular crosslinkages. The theory was advanced that the decreased production of collagen was due to a decrease in fibroblast activity occasioned by a limitation of dietary methionine. This limitation was believed to be the result of the decrease in dietary sulfate which normally would spare methionine from its function in furnishing sulfate for the sulfation of mucopolysaccharides.

The level of inorganic sulfur in an animal's diet has been long thought to be inconsequential (1, 2). The opinion was expressed by one writer (2) that the dietary sulfur amino acids were the principal source of sulfate for the sulfation of sulfomucopolysaccharides in animals.

Recent observations of this laboratory have indicated that inorganic sulfur has a definite role in the diet (3). Gilmore [4] reported that rats fed diets low in inorganic sulfur and containing 0.60% ^{35}S-methionine, incorporated less ^{35}S into cartilage mucopolysaccharides than their counterparts which were fed a higher (100-fold) level of inorganic sulfur. Button et al.[5] reported that cellular lipoproteins isolated from the livers of rats fed low levels of inorganic sulfur contained significantly less hexosamine than their normal controls which was suggestive of a failure in sulfomucopolysaccharide metabolism.

This study was begun to determine whether this apparent failure in mucopolysaccharide metabolism was reflected in a change in collagen metabolism.

EXPERIMENTAL PROCEDURE

Male rats of the Wistar strain were raised from weaning at 21 days of age for 16 weeks with 2 modifications of the basic experimental diet described in the first paper in this series (4). The 2 levels of sulfate, 0.02% (called the normal level) and 0.0002% (called the low level), were furnished by the salt mixture of Hubbell et al. (5) and the low sulfate modification of this salt mixture described previously ((4) table 2). Growth progress was followed by weekly weighing at which time the animals were examined for any gross changes in appearance.

Tissue fractionation and gel formation. At age 134 days, the animals were decapitated, the skins removed promptly and stored at −20° until processing. After the hair, fat and loose connective tissue were removed, the skin was finely minced with scissors. The minced hides were serially extracted with 5 daily changes of 5 volumes (w:v) of 1.0 M sodium chloride solution, and 5 daily changes of 5 volumes (w:v) of 0.5 N acetic acid by shaking at 4°. After extraction, the insoluble residue was washed by centrifugation with 3 changes of 5 volumes (w:v) of 0.10 N sodium hydroxide and 3 changes of 5 volumes (w:v) of distilled water and steam-hydrolyzed in 5 volumes (w:v) of distilled water. The extracts were quantitatively

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[2] Holder of a National Defense Education Act Predoctorial Fellowship during a part of this study.

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TABLE 1

Relative distribution of collagen fractions and total collagen observed in the skins of rats fed at low and normal levels of sulfate

Diet group	Fraction			Total collagen
	Neutral salt-soluble	Acid-soluble	Total soluble	
	g/100 g of collagen			*g/100 g of wet skin*
Normal sulfate [1]	10.5	32.0	42.5	36.8
Low sulfate [2]	6.0	28.7	34.7	24.1
t value [3]	3.833	0.893	1.744	4.698
P value	< 0.01	> 0.50	> 0.10	< 0.01

[1] Three animals in this group.
[2] Five animals in this group.
[3] Student's *t* test.

collected by centrifugation at 21,600 × *g* for 30 minutes at 4° and the amount of hydroxyproline contained in aliquot portions of the extracts determined (6). The amount of collagen in these portions was estimated by multiplying the hydroxyproline content by 7.46 as suggested by Fry et al. (7).

Portions of the neutral salt-soluble and acid-soluble collagen solutions were purified by the method used by Martin et al. (8). The rate of gel formation in neutral salt- and acid-soluble collagen solutions was determined by the method outlined by Hausmann (9) using both crude and purified solutions. Statistical analysis was made by Student's *t* test (10).

RESULTS

During the course of the study there was no statistically significant difference in the rate of growth, as measured by weight gains over the total period, between the experimental groups. No gross abnormalities were noted in either group.

The relative distribution of the soluble collagen fractions is presented in table 1. The skins from the animals fed the low sulfate diets produced significantly less neutral salt-soluble collagen compared with that produced in the skins of animals fed the 0.02% sulfate diet, whereas decreasing the level of sulfate had no statistically significant effect on the amount of acetic acid-soluble collagen produced.

This, coupled with the variability of the amounts of acid-soluble collagen produced as indicated by the low *t* value, accounted for the lack of a statistically significant difference in the amount of total soluble collagen produced in the skins of the low sulfate group compared with their normal controls. There was a highly significant decrease in the total amount of collagen produced per 100 g of wet skin. Whether this decrease in concentration was the result of an accelerated breakdown of collagen pres-

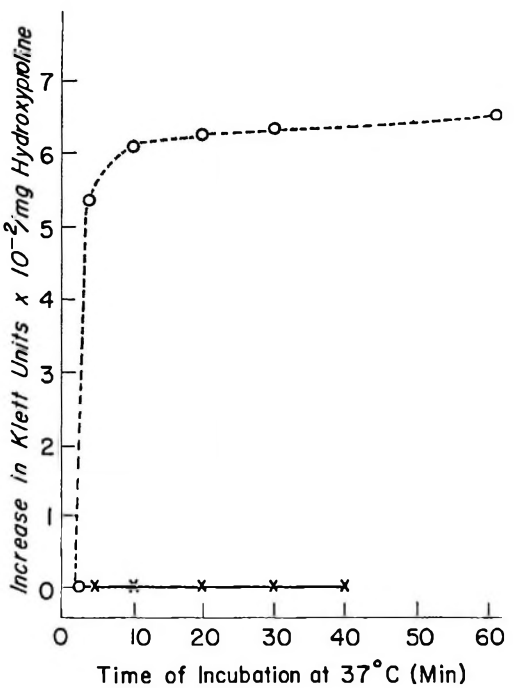

Fig. 1 Rate of gel formation at 37°, pH 7.4, of neutral salt-soluble collagen solutions containing approximately the same amount of hydroxyproline. The curve from the normal-sulfate group is marked as 0 - - - - 0, and the curve from their low sulfate counterparts is marked x——x. The upper curve is the average of 3 animals and the lower curve is the average of 5 animals.

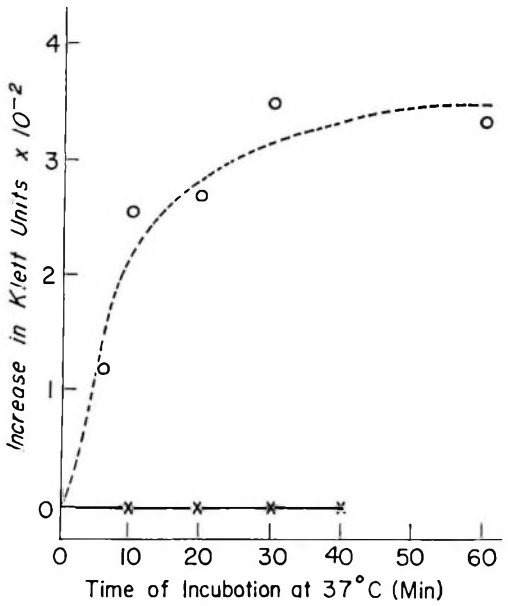

Fig. 2 Rate of gel formation at 37°, pH 7.4, of acid-soluble collagen solutions containing one milligram of hydroxyproline. The upper curve (0----0) is that of the normal sulfate group, and the lower curve (x——x) is that from the low sulfate group. The upper curve is the average of 3 animals, and the lower curve is the average of 5 animals.

ent or of a failure to synthesize collagen is not known; however, the highly significant decrease in the percentage of neutral salt-soluble collagen in the skins of the low sulfate group was suggestive of failure in synthesis.

The results of the gel formation studies are shown in figures 1 and 2. The neutral salt-soluble and acid-soluble collagen solutions derived from skins of animals fed 0.02% of sulfate diets formed gels rapidly, after a brief lag period of about 3 minutes, and gelation was essentially complete in 30 or 40 minutes. The same solutions derived from animals fed the low sulfate diets did not form gels under the same conditions which produced gels in collagen solutions from the control group. The experiments with the collagen solutions from the low sulfate group were not reported beyond 40 minutes because of the appearance of thready clumps in some of the tubes. It was noted, however, that even prolonged incubation (up to 120 minutes) would not produce gels. The same results were obtained using purified preparations but will not be reported here in detail.

DISCUSSION

The fibroblast, which is the cell generally conceded to synthesize both collagen and sulfomucopolysaccharides, is sensitive to several factors including the level and quality of the dietary protein. Pearce et al. (11) have reported a decreased fibroblast population in healing experimental wounds inflicted on protein-depleted animals. This decreased population was accompanied by a decrease in the production of collagen and stainable mucopolysaccharides. Deprivation of methionine has been reported (12) to block the maturation of the fibroblast and concomitantly, collagen production.

It has been suggested by Kun (2) that dietary methionine is the principal if not the sole source of inorganic sulfate for the sulfation of sulfomucopolysaccharides. Gilmore [6] observed that the 0.60% methionine contained in some experimental diets was not sufficient to furnish sulfate for the sulfation of mucopolysaccharides if the inorganic sulfur in the diet was decreased to a low level. In effect, methionine could be thought of as a limiting amino acid, and as a consequence the production of collagen could have been impaired. Alternatively, the decreased amount of collagen present in the skins of animals fed the low sulfate diets may have been the result of an increased catabolism of collagen already present. The writers, however, do not believe this to be the case, since, as Peacock (13) pointed out, the level of neutral salt-soluble collagen present in the healing wound was a good measure of the rate of collagen synthesis. The fact that the animals had been fed the sulfate-deficient diet since weaning also argues against a breakdown of collagen already formed.

It is obvious from the gelation studies that the soluble collagen from the skins of animals fed the low sulfate diets is not normal. Wood (14, 15) fractionated neutral salt-soluble collagen into 3 fractions: A, B and C. These fractions varied with respect to their subunit composition and their role in gel forming. Fraction A contained α- and β-units in the ratio of 4:1,

[6] See footnote 4.

fraction C had an α- to β- ratio of 2:1, whereas the B fraction contained no β-component. The C fraction, which contained the greatest proportion of the more mature, crosslinked, β-component was the so-called nucleating fraction about which the fibrils of the gel formed. The B fraction which contained no β-component would not form thermal gels. The β-components are formed from α-components by intramolecular crosslinkage (15).

It appears then that there was either a lack of the nucleating fraction or a large amount of the B fraction was present in the collagen solutions from the skins of animals fed at low levels of inorganic sulfur. While precisely which is the case is unknown, in either event a decrease in intramolecular crosslinkage appears to be indicated.

Why decreasing the level of dietary sulfate should cause a change in crosslinkage is not known; however, a tentative suggestion may be made. As pointed out above, there is reason to believe that decreasing the level of dietary sulfate impedes the activity of the fibroblast. It is known that in addition to synthesizing collagen, this cell also secretes sulfomucopolysaccharides. Button et al.[7] reported that decreasing the level of inorganic sulfur in the diet caused a significant decrease in the hexosamine bound to cellular lipoproteins which are thought to be the membranous material of the cell (16). Although the role of mucopolysaccharides in collagen synthesis and maturation is ill-defined, Lowther (18) suggests that the heavy mucopolysaccharide covering of the fibroblast serves to hold the newly secreted collagen fibers so that crosslinkage and maturation can occur. Wood and Keech (19) suggest that sulfated mucopolysaccharides participate in the formation of the nucleating fraction. It is reasonable, therefore, to suggest that the inability to form gels of the collagen solutions from skins of animals fed low sulfate diets may be traced to abberations in sulfomucopolysaccharide metabolism.

The acid-soluble fraction was found to be homogeneous by Wood (14) and composed of more mature, crosslinked collagen as marked by increased amounts of the β-subunit (16). It appears that even this more mature collagen fraction is abnormal in the animals fed the low sulfate diets. The precise nature of the abnormality is the subject of continuing investigation.

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Effect of Acetate and Propionate on the Utilization of Energy by Growing-Fattening Lambs [1]

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ABSTRACT Growing-fattening lambs fed a basal ration of pelleted alfalfa hay showed no response in either rate of gain in body weight or feed required per unit gain when propionate or acetate was incorporated in the feed. The addition of corn to the basal ration resulted in a significantly faster rate of gain. Estimates of the composition of gains were obtained by slaughter and chemical analysis of 4 lambs at the beginning, and 2 lambs from each treatment group at the end of the trial. The gains of the lambs fed propionate, acetate or corn were considerably higher in fat than those of controls. On an energetic basis, these groups converted digestible energy to body gain with considerably greater efficiency than the control group. No difference in efficiency was observed among supplements.

Calorimetric studies of Armstrong and Blaxter (1) and Armstrong et al. (2) have indicated clearly that propionic and butyric acids are utilized with greater efficiency than acetic acid when infused into the rumen of fattening sheep. On the basis of this work it might be postulated that an increase in either rate of gain or feed efficiency, or both, would result from the addition of propionic or butyric acid to rations which normally yield high proportions of acetic acid during ruminal fermentation. In general, however, addition of the free acids to rations has rendered them unpalatable (3, 4) and the acids when added as salts have not resulted in significant differences in rates of body gain in lambs (3, 5, 6). In beef cattle the addition of 2% sodium propionate to fattening rations did not improve gains over controls, although it tended to reduce feed intake and increase feed efficiency slightly (7). Intra-ruminal infusions of acetic, propionic and butyric acids in non-lactating dairy heifers fed a normal ration resulted in increased nitrogen retention and body weight gain, but in no case was the difference among acids significant (8).

The possibility remained that dietary propionate or butyrate might result in a greater gain of body fat than acetate, without influencing rate of gain in weight. In the experiment reported here complete carcass analyses were made to compare the composition of gains in lambs fed a basal roughage ration with or without additions of acetate, propionate or corn.

EXPERIMENTAL PROCEDURE

Sixty-three native (New York State crossbred) shorn, feeder lambs (23 to 35 kg) were divided as to sex and initial weight and randomly assigned to 9 groups of 7 lambs each. The groups were then allotted at random to 4 dietary treatments (2 groups per treatment). Four lambs, representing the weight range, from the remaining group were killed for analysis at the beginning of the trial. The 3 remaining lambs in this group were discarded. Each group was assigned a separate pen and group-fed twice daily on an ad libitum basis for a 50-day feeding period. Feed consumption and refusals were recorded daily and body weights to the nearest 0.2 kg were taken weekly. Water and trace-mineralized salt were available throughout the trial. At the end of the trial, one representative lamb from each pen (2 per treatment) was selected, shorn, and killed for analysis.

The 4 experimental rations employed were: 1) alfalfa hay, 2) alfalfa hay plus 113 g shelled corn per lamb per day, 3) alfalfa hay containing 10% sodium propionate ($NaC_3H_5O_2$), and 4) alfalfa hay con-

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taining 10% sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$).

The feeds were prepared and fed in complete pelleted form, except for the shelled corn, which was added in appropriate quantity at each feeding. Alfalfa hay, the basal portion of all rations, was ground in a hammermill through a 0.3-cm screen. A portion of this was pelleted in a mobile pelleting unit through a 2-cm die. After repeated, unsuccessful efforts with the mobile machine to pellet the mixture of ground hay and sodium acetate or propionate, this material was pelleted in a commercial feed mill through a 0.6-cm die. As a result of these difficulties the basal pellets used in the trial were of a different diameter than those containing the propionate and acetate, although all were made from the same lot of ground hay. Following the growth trial, 4 of the lambs were used to assess the digestibility of the 4 rations.

Feed samples were composited during the growth trial and during each period of the digestion trials. These, together with the dried fecal samples were subjected to proximate analysis and heats of combustion were determined in a Parr adiabatic oxygen bomb calorimeter. The shorn, complete empty bodies of the lambs killed were frozen, ground, mixed and sampled in duplicate for analysis. These samples were freeze-dried to constant weight for moisture determination and subsequently extracted with diethyl ether. The dried extracted material was analyzed for moisture, ether extract, nitrogen and ash, and heats of combustion were determined as described above. Energy content of the empty bodies was computed by assigning a value of 9.405 kcal/g [3] to the extracted fat, and using the heats of combustion of the dried "fat-free" material. The initial composition and energy content of the ingesta-free, wool-free bodies of each group of lambs were estimated by computing the mean composition and caloric value per unit live weight of the 4 lambs initially slaughtered and applying these values to the mean initial weights of the groups. Final composition and energy content were estimated in the same manner. The composition and energy of the wool were not estimated, although wool weights were recorded.

RESULTS AND DISCUSSION

Proximate composition of the feeds used is presented in table 1 and body weight gains and feed consumption data in table 2. The ash values for the feeds containing acetate or propionate were higher than would be expected from the added sodium. They were, however, consistent among feed samples and may reflect the partial oxidation of sodium in the ash determination. The lambs receiving alfalfa hay and corn gained significantly ($P < 0.01$) more body weight than those on the other treatments. Average body weight gains of those on the basal, acetate and propionate treatments were almost identical. This observation is in substantial agreement with earlier work (5). An examination of the data on gastrointestinal "fill" of the lambs killed, indicated only small variation among treatments (range 21 to 24% of live weight). Wool weights likewise showed

[3] Paladines, O. L. 1963 Energy utilization by sheep as influenced by physical form, composition and level of intake of diet. Ph.D. Thesis, Cornell University.

TABLE 1

Proximate composition of feeds

Feed	Water	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract	Ash	Organic matter
	%	%	%	%	%	%	%
Alfalfa pellets	12.1	14.0	1.8	32.5	34.1	5.6	82.4
Alfalfa + propionate [1]	15.4	12.8	1.7	28.8	30.7	10.6	74.0
Alfalfa + acetate [2]	16.4	12.8	1.7	29.6	30.6	8.9	74.7
Shelled corn	13.2	8.8	4.5	2.4	70.0	1.1	85.7

[1] Sodium propionate N.F. ($NaC_3H_5O_2$) added to ground hay at 10% level; propionic acid equivalent approximately 7.7% of the feed.
[2] Sodium acetate N.F. ($NaC_2H_3O_2 \cdot 3H_2O$) added to ground hay at 10% level; acetic acid equivalent approximately 4.4% of the feed.

little variation (range 540 to 650 g). Slightly less feed was consumed by the lambs on the acetate treatment, and particularly those receiving the propionate treatment, than by the control or corn groups. This may be attributable to the higher sodium content of these diets (4). The average calculated consumption of acetic and propionic acids in the rations was very nearly equal on a molecular basis; on a caloric basis, however, the lambs on the propionate treatment consumed in the form of organic acid almost twice as much energy as those on the acetate treatment.

The addition of acetate or propionate to the rations had little effect on the digestion coefficients for any of the proximate components (table 3), with the possible exception of crude fiber. Acetate appeared to reduce crude fiber digestibility slightly, but the reduction was not statistically significant. The total digestible nutrient (TDN) values for the feeds containing acetate and propionate were substantially lower than the TDN value of the basal feed primarily because of their higher ash content. On an ash-free basis the TDN values were 53.8, 53.4 and 50.9 for the basal, propionate and acetate treatments, respectively. The digestion coefficients for the shelled corn were obtained in "difference" trials, in which corn was fed at low levels with the basal pelleted ration. The coefficients for corn were extremely variable among animals as is frequently the case in such trials; however, the average values are in reasonably good agreement with those for No. 2 dent corn as listed by Morrison (9). The average TDN value is very close to Morrison's value on a dry-matter basis and was, therefore, considered valid for use in efficiency calculations.

The data in table 4 show that the lambs from the propionate, acetate or corn treatments were fatter than those from the basal ration. This is reflected in both the composition and energy content of the dry matter of their ingesta-free, wool-free bodies. Furthermore, there was essentially no difference in composition of those on the acetate and propionate treatments. While

TABLE 2

Average body weight gains and feed consumption

	Ration			
	Basal	Propionate	Acetate	Corn
No. of lambs	14	14	14	14
Initial weight, kg	27.7	28.0	28.1	27.6
Final weight, kg	35.7	35.4	35.6	37.3
Daily gain, kg [1]	0.16	0.15	0.15	0.19
Feed consumed, kg	87.6	79.4	84.3	86.8
TDN [2] consumed, kg	38.8	31.4	32.1	40.5
DE [3] consumed, megcal	191.6	181.9	177.7	197.6

[1] SE of mean = 0.012. Corn > others ($P < 0.01$).
[2] Total digestible nutrients.
[3] Digestible energy.

TABLE 3

Digestion coefficients, total digestible nutrient (TDN) and digestible energy (DE) values of the feeds

Feed	Dry matter	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract	Energy	TDN [1]	DE
	%	%	%	%	%	%	%	*kcal/kg*
Alfalfa pellets	51.2 [2]	65.6	49.6	33.8	64.9	52.6	50.4	2487
Alfalfa + propionate	52.9	66.7	44.3	32.5	64.2	55.8	46.7	2709
Alfalfa + acetate	50.1	64.4	46.7	30.4	62.7	52.5	45.5	2521
SE [3]	±1.02	±1.40	±5.71	±1.38	±1.38	±0.99	±0.93	±25
Shelled corn [4]	89.1	73.8	85.0	50.4	93.7	87.7	92.8	4095

[1] Dry-matter basis.
[2] Each coefficient is the average of 3 observations.
[3] SD of a mean.
[4] Values determined by difference. The standard errors are not appropriate to the values for shelled corn.

those receiving corn gained more weight and thus had a higher total body energy content than those on the fatty acid treatments, they were not appreciably different in composition. Those fed the basal ration, however, were similar in composition to the lambs analyzed at the beginning of the trial. Bentley et al. (5) presented data on the moisture and fat content of the ground shoulders from lambs fed acetate or propionate which, though quite variable, suggested that those fed the acid salts were fatter than controls.

The estimated composition of the gains, as well as the estimated total energy gains for the entire groups are presented in table 5. These data suggest that the gain in body weight of the basal group was much higher in water and lower in fat than that of the other 3 groups, all of which were very similar. The validity of these data is obviously predicated on the assumption that the lambs analyzed were typical of the lambs in the groups they represented. While compositional data are, of course, unavailable to support this assumption, the average rate of body weight gain of the 2 lambs slaughtered in each case closely paralleled the average rate of gain of their respective treatment groups.

Calculations of efficiency of gain (table 6) indicate that the feed required per unit of gain in live weight was similar for the basal, acetate and propionate rations, and somewhat lower for the ration containing corn. When the caloric efficiency was computed on the basis of digestible energy (DE) required per unit energy gain, the basal ration was less efficiently utilized than the other three, all of which were similar. Digestible energy intakes were very similar with the acetate and propioate treatments. The fact that the lambs consumed nearly twice as many calories from propionate as they did from acetate plainly suggests that the propionate was not utilized with greater energetic efficiency than the acetate.

The lack of agreement between these results and those of Armstrong et al. (1, 2) is not readily explained. Several factors, however, may have relevance. The animals used by Armstrong et al. (1, 2) were adult sheep; those in the present study

TABLE 4

Average chemical analyses of ingesta-free, wool-free lamb bodies

Treatment	No. lambs	Dry matter	Composition, dry-matter basis			
			Crude protein [1]	Ether extract	Ash	Total energy
		%	%	%	%	*megcal*
Initial	4	38.0	42.4	48.1	11.9	54.6
SE [2]		±1.2	±2.0	±2.7	±0.7	±4.24
Final						
Basal	2	35.5	43.6	46.7	10.4	65.1
Basal+propionate	2	38.8	39.1	52.0	9.4	76.8
Basal+acetate	2	38.6	39.6	51.6	9.9	76.0
Basal+corn	2	38.4	38.5	53.0	9.4	80.5
SE		±1.7	±2.9	±3.8	±1.0	±5.99

[1] N × 6.25.
[2] SD of a mean.

TABLE 5

Estimated composition and total energy of ingesta-free, wool-free body gains

Treatment	Water	Fat	Protein	Ash	Total energy
	%	%	%	%	*megcal*
Basal	73.8	11.0	14.0	1.3	11.7
Basal+propionate	60.9	26.0	12.2	1.0	21.6
Basal+acetate	60.5	24.7	13.1	1.8	21.7
Basal+corn	61.2	25.3	12.0	1.5	27.1

TABLE 6

Efficiency of feed utilization

Criteria	Treatment			
	Basal	Basal + propionate	Basal + acetate	Basal + corn
Feed/kg gain, kg	11.0	10.6	11.4	8.9
TDN [1]/kg gain, kg	4.9	4.2	4.3	4.2
TDN/megcal gain, kg	3.32	1.45	1.48	1.49
DE [2]/megcal gain, megcal	16.38	8.42	8.19	7.29
Net utilization of DE for gain,[3] %	11.7	24.0	25.4	26.0

[1] Total digestible nutrients.
[2] Digestible energy.
[3] Retention of energy surplus to maintenance; maintenance requirement estimated as 76 $W^{0.75}_{lb}$ (Garrett et al. (11)). Energy gain in wool disregarded.

were growing-fattening lambs. Using growing heifers infused with fatty acids, Rook et al. (8) failed to obtain a significant difference between acetic and propionic acids in calculated energy gain. In the present study the acids were incorporated in the feeds as acid salts, whereas in the British work they were infused as dilute acids into the rumen. Although the acid salts did not appear to grossly alter digestibility, their possible effect on the rumen environment should not be overlooked as a factor contributing to the difference in results. It is interesting in this respect that Bentley et al. (5) reported a lower total amount of volatile fatty acids in the rumens of lambs fed propionate than of those fed acetate.

The net utilization of DE surplus to maintenance was computed (table 6) using the formula of Garrett et al. (11) to estimate maintenance requirements. When expressed in this way the efficiency of energy utilization is again very similar for the 3 supplemented groups. Approximately one-half of the DE consumed was required for maintenance. The evidence available (10) indicates that mixtures of volatile fatty acids of widely varying proportions are utilized with essentially equal efficiency for this purpose. It may be that the present study was not sufficiently sensitive to detect differences between the acetate and propionate rations at the levels employed.

The striking difference in efficiency between the basal and supplemented groups is also difficult to explain. The basal group consumed more DE than either the acetate or propionate groups. If it is assumed that the added acetate and propionate were quantitatively absorbed without the energy losses normally accompanying ruminal fermentation, they would account for approximately 7 and 13%, respectively, of the DE consumed. The elimination of the energy loss associated with fermentation of such a small portion of the caloric intake appears to be much too small to account for the marked change in energy gain. Bentley et al. (5), however, reported very high feed replacement values for acetate and propionate in their studies. The results of the present experiment indicate that acetate, propionate or corn when added to an all-hay ration greatly increase the efficiency with which energy is stored in the growing-fattening lamb.

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Effect of Physical Form, Composition and Level of Intake of Diet on the Fatty Acid Composition of the Sheep Carcass [1]

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ABSTRACT The fatty acid composition of the mixed lipids was determined in the ground, whole carcasses of 45 sheep, each of which had ingested for 196 days one of 3 diets at one of 3 levels of intake. The sheep were of 2 ages (27 and 15 months at the time of slaughter). The proportions of the major fatty acids were not different for the sheep ingesting the 3 diets (chopped hay; pelleted, ground hay; and a pelleted mixture of 45% of corn meal and 55% of ground hay). There was, however, a significantly lower proportion of heptadecanoic and heptadecenoic acids in the carcasses of sheep fed the corn-containing diet than in those ingesting the other diets. Carcasses of sheep on the low level of intake contained 18% more of oleic acid than did those of sheep receiving the high level of intake. This significant increase ($P < 0.05$) was at the expense mainly of stearic acid. The carcass lipids of 27- and 15-month-old sheep adjusted to the same body weight had essentially the same fatty acid composition. The proportions of branched heptadecanoic acid were significantly greater ($P < 0.05$) in the 27-month-old sheep. In this study, body weight was the most important factor influencing the fatty-acid composition of the carcass. The percentage of oleic acid and the weight of the ingesta-free body were significantly ($P < 0.05$) correlated. As body weight increased, the percentage of oleic acid increased. This increase was compensated by a decrease in the concentrations of stearic, palmitoleic, odd-numbered and branch-chain fatty acids.

The fatty acid composition of adipose tissue in ruminants is relatively difficult to alter by changing the nature of the dietary lipids. It has been amply demonstrated that biohydrogenation of dietary unsaturated fatty acids by the rumen microflora (1) [3] accounts for this constancy in body fat composition. However, in commonly fed sheep diets, it is dietary carbohydrate rather than dietary lipid that is the main precursor of adipose tissue fatty acid. The objectives of the present experiment were to study the effects on the fatty composition of the adipose tissue of sheep of (a) diets known to produce different proportions of end-products of carbohydrate digestion; (b) level of intake of the diet; and (c) age and body weight of the animals.

EXPERIMENTAL PROCEDURE

Forty-five sheep (eighteen, 8-month-old and twenty-seven, 20-month-old sheep) were used in this study. During 4 months prior to the experiment the animals were fed calculated amounts of feeds in the hope of obtaining the same body weights within each age group. The 45 sheep were then assigned at random to the treatments of a factorial experiment with 3 levels of intake and 3 diets superimposed on 2 ages. The 3 diets fed were: chopped hay; pelleted, ground (1.6-mm sieve) hay from the same harvest; and a pelleted mixture of 45% corn meal and 55% of the same ground hay. The hay, constituted mainly of timothy, was harvested on June 4.

Each diet was fed at 3 levels characterized as follows: low (sufficient to produce a slight positive weight balance); medium (intermediate between low and high); and high (ad libitum). At each the low and medium levels of intake, the chopped and pelleted hay diets were fed in equal amounts of dry matter per unit of metabolic size (i.e., body $\text{weight}_{kg}^{0.734}$). A more detailed description of the diets, levels of

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[3] Reiser, R. 1951 Hydrogenation of polyunsaturated fatty acids by the ruminants. Federation Proc., *10:* 236 (abstract).

intake and changes in the gross chemical constituents of the sheep bodies have been published (2).

At the completion of the 196-day feeding period the sheep were slaughtered and the carcasses immediately ground in a 25-hp high capacity grinder mounted with a 9.5-mm front plate. The material obtained was reground 5 times to insure mixing. Within 2 hours duplicate 20-g samples were extracted for 5 minutes in a blender with a 2-to-1 chloroform-methanol mixture (3). The chloroform-methanol lipid extracts, after having been washed with an amount of 0.02% calcium chloride solution equivalent to 20% of their volume, were dried under nitrogen and stored in hexane at 20°. About 300 mg of lipid material were saponified at 80° for one hour. The hydrolysate was then washed with hexane and acidified. This was followed by fatty acid extraction with ethyl ether. Methylation was carried out by the boron trifluoride method (4). The methyl esters were separated by gas chromatography on a 1-m column packed with 10% ethylene glycolsuccinate on 100 to 120-mesh silanized gas-chrom P.[4] The operating conditions were as follows: column temperature = 160°; carrier gas (helium) flow rate, 70 ml/min; hydrogen flow rate, 70 ml/min; oxygen flow rate, 55 ml/min. The weight percentage of the fatty acid components was obtained by measurement of peak areas. Quantitative results with commercial standards [5] agreed with the theoretical composition with a relative error less than 10% for minor components (< 10% of total mixture) and less than 4% for major components (> 10% of total mixture).

The peaks were tentatively identified by considering the information provided by the following procedures: (a) The carbon numbers (5) of the unknown peaks were compared with those of reference fatty acid methyl esters; (b) the changes in relative size of the various peaks upon hydrogenation (6) were evaluated; and (c) acetoxymercuri - methoxy-derivatives were prepared and separated into classes according to unsaturation by means of thin-layer chromatography (7) and the adducts of esters were recovered and separated by gas chromatography. More details concerning the identification of the fatty-acid composition of sheep body lipids will be published later.

RESULTS AND DISCUSSION

The sheep lipid fatty-acid composition observed in this study corroborates the general statement that ruminant adipose tissues are relatively constant in composition and rather resistant to changes when various dietary treatments are imposed. Even though many of the observed differences were mathematically significant, most of them do not appear to have much biological significance. The mean fatty acid composition of the 45 sheep included in the study is presented in table 1. These means are not corrected for differences in body weight.

TABLE 1

Proportions of fatty acids in the lipid fraction of 45 sheep carcasses

Fatty acid	Means
	%
8:0	0.1 ± 0.04 [1]
10:0	0.1 ± 0.04
12:0	0.2 ± 0.04
14[B] [2]	0.2 ± 0.04
14:0	3.0 ± 0.6
14:1	0.4 ± 0.1
15[B]	0.5 ± 0.1
15:0	1.0 ± 0.2
16[B]	0.5 ± 0.1
16:0	23.8 ± 1.7
16:1	3.1 ± 0.3
17[B]	1.1 ± 0.3
17:0	1.8 ± 0.2
17:1	1.0 ± 0.2
18:0	22.3 ± 2.3
18:1	36.8 ± 2.4
19:0	0.4 ± 0.2
18:2	2.0 ± 0.4
18:3	1.5 ± 0.2
20:1	0.2 ± 0.1

[1] Mean ± SD.
[2] [B] indicates branched fatty acids.

Effect of diet on carcass lipid composition. The proportions of the major fatty acids (i.e., those present in concentrations greater than 10%) in the mixed lipids were not significantly different in the carcasses of sheep ingesting the 3 diets (table 2). Numerous studies have shown that the proportion of propionic acid absorbed increases as the quantity of soluble carbo-

[4] Applied Science Laboratories, Inc., State College, Pennsylvania.
[5] See footnote 4.

hydrates increases in the diet. Propionic acid has been demonstrated in vitro (8, 9) to be an odd-numbered fatty acid precursor. On this basis it would be expected that larger proportions of odd-chain acids would occur in the lipids of sheep fed the corn pellet diet. However, the proportions of these acids for the 3 dietary treatments were not greatly different. Contrary to the expected trend, there was a significant ($P < 0.05$) decrease in the proportions of heptadecanoic (17.0) and heptadecenoic (17.1) acids in the lipids of sheep ingesting the corn-containing diet. The present data support those of Masoro and Porter (10) obtained with rats. Their in vivo studies with propionate-1-^{14}C demonstrated that propionate was incorporated in the adipose tissue as acetyl-CoA following decarboxylation. A negligible proportion of the propionate was incorporated as a 3-carbon unit. Mead and Lewis (11) presented evidence that supports the theory that in the intact rat brain odd-chain acids are the degradation products of even-chain acids by hydroxylation and oxidative decarboxylation. The preceding experimental results are a reminder that the existence of a synthesizing system identified in vitro does not necessarily reflect its quantitative importance in the whole animal.

Effect of level of intake. Sheep on the low level of intake had a significantly higher ($P < 0.05$) proportion of oleic acid than those receiving the high level of intake (table 3). This decrease in oleic acid proportion with increased level of intake is accompanied by a significant decrease ($P < 0.05$) in the proportion of palmitic acid. The medium level was intermediate between the low and high levels of intake for the differences just mentioned. A valid explanation for this effect of level of energy intake is not apparent to the investigators. The proportion of absorbed energy utilized for maintenance decreases as the level of intake increases. If certain absorbed metabolites were used preferentially to others for energy-yielding oxidative pathways, this could mean that the proportions of metabolites available for lipid synthesis would be modified with the level of energy intake.

TABLE 2

Effect of various diets on the fatty acid composition of sheep carcass

Diet	Fatty acid [1]																			
	8:0	10:0	12:0	14[B]	14:0	14:1	15[B]	15:0	16[B]	16:0	16:1	17[B]	17:0	17:1	18:0	18:1	19:0	18:2	18:3	20:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Chopped hay	0.1	0.1	0.2	0.2	3.6	0.6	0.6	1.0	0.6	22.6	3.3	1.2	2.1	1.3	20.8	37.4	0.4	1.9	1.6	0.2
Pelleted hay	0.1	0.1	0.2	0.2	2.9	0.4	0.5	0.8	0.6	24.3	2.9	1.0	1.8	1.0	22.7	36.0	0.4	2.2	1.7	0.2
Corn-hay pellets	0.1	0.1	0.1	0.1	2.6	0.3	0.5	0.8	0.4	24.3	3.0	1.0	1.7	0.9	23.4	37.0	0.5	2.0	1.2	0.2

[1] The fatty acid composition has been corrected by covariance analysis for differences in body weights. For a given fatty acid, percentages underlined are not significantly different at the 5% level of probability.

TABLE 3

Effect of level of intake on the fatty composition of the sheep carcass

Level of intake	Fatty acid [1]																			
	8:0	10:0	12:0	14[B]	14:0	14:1	15[B]	15:0	16[B]	16:0	16:1	17[B]	17:0	17:1	18:0	18:1	19:0	18:2	18:3	20:1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Low	0.1	0.1	0.2	0.2	3.4	0.5	0.4	0.7	0.5	22.0	2.9	0.9	1.7	1.0	21.4	39.9	0.3	2.1	1.6	0.2
Medium	0.1	0.1	0.2	0.2	3.3	0.5	0.6	0.9	0.5	23.3	3.2	1.1	1.8	1.1	22.0	36.9	0.4	2.1	1.6	0.2
High	0.1	0.1	0.1	0.2	2.5	0.4	0.6	0.9	0.5	25.8	3.2	1.1	2.2	1.1	23.6	33.7	0.5	1.9	1.4	0.2

[1] The fatty acid composition has been corrected by covariance analysis for differences in body weights. For a given fatty acid, percentages underlined are not significantly different at the 5% level of probability.

TABLE 4

Effect of age on the fatty acid composition of sheep carcass

Age	Fatty acid [1]																			
	8:0	10:0	12:0	14[B]	14:0	14:1	15[B]	15:0	16[B]	16:0	16:1	17[B]	17:0	17:1	18:0	18:1	19:0	18:2	18:3	20:1
months	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
27	0.0	0.1	0.1	0.2	2.6 [2]	0.4	0.6	0.9	0.5	23.3	3.3	1.4	1.9	1.1	23.4	36.5	0.5	1.8	1.3	0.2
15	0.1	0.1	0.2	0.2	3.5	0.5	0.6	0.9	0.5	24.1	2.9	0.8	1.8	1.1	21.2	37.1	0.3	2.3	1.7	0.2

[1] The fatty acid composition has been corrected by covariance analysis for differences in body weights. For a given fatty acid, percentages underlined are not significantly different at the 5% level of probability.

[2] Significantly different at 10% level of probability.

Effect of age. The carcass lipids of 27-month- and 15-month-old sheep adjusted by covariance to the same body weight had essentially the same fatty acid composition (table 4). The proportions of branched heptadecanoic acid, 17 [B], were larger in the 27-month-old sheep. Isovaleric, isobutyric and 2-methylbutyric acids have been shown to be produced from valine, leucine and iso-leucine by bacterial deamination and decarboxylation in the rumen (12, 13). These branched short-chain fatty acids are the precursors of long-chain fatty acids with branched carbon skeletons. It is then expected that branched-chain fatty acids would be more abundant in the adipose tissue of older sheep because they have had a functional rumen for a longer time.

There was a trend for the carcass lipids of the 15-month-old sheep to contain a greater proportion of short-chain fatty acids. The origin of part of these short-chain fatty acids might possibly be traced to milk fatty acids which have persisted after weaning.

Effect of body weight. In most studies concerned with the effect of the nature of the diet on the fatty acid composition of adipose tissue, it has been assumed that diet is the only variable to account for the changes in lipid composition that might ensue. In this particular study, body weight was an important factor influencing the fatty acid composition of the adipose tissue. Simple correlation coefficients between the major fatty acid percentages and the empty body weight (ingesta-free body weight) of sheep are presented in table 5. Oleic acid percentage increased (fig. 1) with increasing body weight. This increase is mainly at the expense of stearic, palmitoleic, odd-numbered and branched-chain fatty acids. It is probable that in some experiments with ruminants, the adipose tissue composition changes attributed to manipulations in dietary composition might have been partly explained on the basis of differences in body weight between the various treatment groups upon completion of the feeding period. Shaw et al. (14) reported a 20% increase in iodine number of the visceral fat attribut-

TABLE 5

Correlation coefficients between various fatty acid percentages and empty-body weight

Fatty acid [1]	Mean	Correlation [2]
	%	
14:0	3.0 ± 0.6 [3]	0.20
16:0	23.8 ± 1.7	−0.11
16:1	3.1 ± 0.3	−0.27
18:0	22.3 ± 2.3	−0.29
18:1	36.8 ± 2.4	0.38*
O–N	4.8 ± 1.0	−0.38*
[B]	2.3 ± 0.6	−0.36*

[1] O–N indicates the sum of the odd-numbered fatty acid percentages and [B] the sum of the branch chained fatty acid percentages.
[2] Simple correlation coefficients.
[3] Mean ± SD.
* Coefficient significant at 5% level of probability.

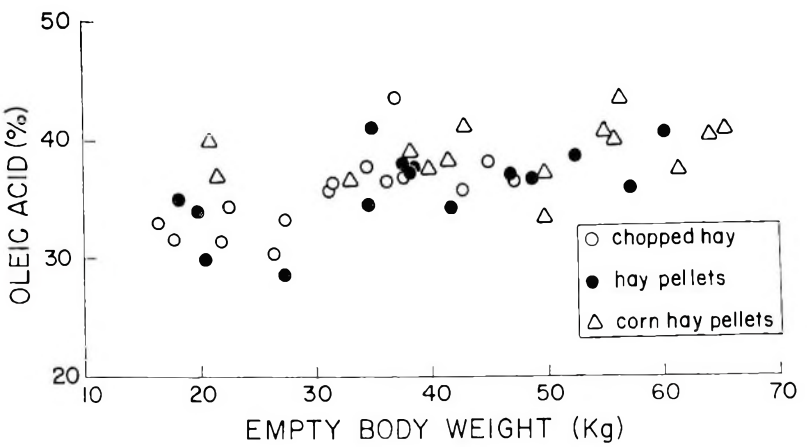

Fig. 1 Relationship between oleic acid contents and empty-body weights of 45 sheep carcasses.

able to the grinding and pelleting of hay and to the inclusion of corn flakes in the diet of cattle. In that experiment the steers of the treatment group gained weight 22% faster than the control animals. Many hypotheses could be considered to explain these data. One possibility is that a lower oxidation rate of the depot fat triglyceride oleic acid relative to other fatty acids would cause an indirect increase of oleic acid with increasing body weight. Gellhorn et al. (15) have reported a similar increase in proportion of oleic acid with increased body weight, in the epididymal fat of the rat. The significant relationship between oleic acid percentage and body weight was observed in the present experiment with sheep fed high-carbohydrate diets (i.e., requiring high *de novo* fatty acid synthesis). Whether this relationship would hold true for high-fat diets remains to be investigated.

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