Changes in Plasma Proteins Associated with the Anemia Produced by Dietary Cadmium in Japanese Quail [1]

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ABSTRACT Day-old Japanese quail of each sex were fed a purified soybean protein diet containing 75 mg zinc/kg, alone or in combination with 75 mg cadmium/kg. After 4 weeks, changes in plasma proteins were characterized by quantitative disc gel electrophoresis, immunoelectrophoresis, and ^{59}Fe labeling and autoradiographic techniques adapted to both types of electrophoresis. Quail receiving cadmium grew more slowly and had a higher mortality than control birds receiving the same adequate level of zinc, whereas both groups had similar feather scores. About 80% of the cadmium-fed birds developed severe anemia. It was associated with markedly increased concentrations of plasma transferrin (15.0% for control birds versus 23.2% for cadmium-fed birds) and moderately decreased levels of plasma albumin (30.9 versus 27.1%) and band 7 (28.6 versus 23.3%), the principal lipoprotein fraction of the plasma. The amount of total plasma protein remained unchanged; thus the increase in transferrin was largely at the expense of band 7. The fractional changes gave rise to markedly elevated transferrin-to-albumin ratios in the cadmium-fed birds (0.49 versus 0.86). None of these birds exhibited an abnormal serum component.

Cadmium toxicity in human beings has long been a recognized hazard by industrial hygienists concerned with the health of individuals working in certain parts of the metallurgical industry. More recently, evidence has been presented that adverse effects of cadmium may be of more widespread significance in certain chronic diseases than had been suspected (1, 2). Most studies of cadmium toxicity have focused on the metal per se, with little concern about the dietary intake and metabolism of related elements. Many of the known effects of cadmium involve the blood and cardiovascular system. It was considered pertinent to investigate effects of cadmium upon changes in the plasma proteins, particularly because these changes can reflect metabolic alterations in various tissues of the body.

Ingestion of cadmium by rats was shown to produce severe anemia (3, 4). In a study of chronic effects of injected cadmium, Truhaut and Boudene (5) observed apparent decreases in the α-globulins with a steady increase in the β-globulins. In a brief note, Lawford (6) reported that young albino rats fed small amounts of soluble cadmium salts had elevated levels of transferrin in their sera.

Cadmium is a recognized metabolic antagonist of zinc. Because the young Japanese quail has been shown to be sensitive to zinc deficiency which resulted in aberrations of the plasma protein patterns under certain conditions (7–9), it was thought that this would be a suitable animal in which to investigate the adverse effects of cadmium. The present experiments were designed to characterize and quantitate the effects of cadmium upon plasma proteins.

EXPERIMENTAL

One-day-old Japanese quail *(Coturnix coturnix japonica)* of each sex were housed in stainless steel cages suspended in stainless steel racks covered with nonwoven cloth and polyethylene film to minimize

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possible environmental contamination with trace-element bearing dust. Continuous control of light and temperature was maintained by regulating the current and size of incandescent lamps in each cage. Groups of 8 to 20 birds each were fed purified diets and demineralized water ad libitum. The birds were wing-banded on day 7 and then were weighed weekly throughout the subsequent 3 weeks of the experiment. Feather scores were noted weekly on each bird after the second week. Perfect feathering was scored as 100. The purified diet, containing 35% soybean protein, was the same as that described previously (7) except that 0.02% ethoxyquin was added to all diets. Most of the soybean protein was rendered low in zinc [4] by the sodium-EDTA extraction method of Davis et al. (10). A total of 75 mg zinc/kg diet was supplied by the soybean protein and by zinc carbonate. The total iron content of the diet was 106 mg/kg as determined by atomic absorption spectrophotometry; 33 mg iron/kg were supplied by ferric citrate. Cadmium was not detectable in the control diet by atomic absorption spectrophotometry ($<$ 0.02 μg/g). Cadmium, 75 mg/kg diet, was fed as the chloride, and provided a zinc–cadmium mole ratio of 1.7:1.

Microhematocrits on nonfasted birds were measured on blood collected from the wing vein. Heparinized blood was collected by cardiac puncture and the separated plasma was stored in acid-washed 1-ml polycarbonate tubes at −15°.

Each plasma sample was analyzed for total protein by the method of Lowry et al. (11). Proteins in 3 μliters of plasma were resolved in 7.5% polyacrylamide gel by the general methods described by Davis [5] (12). For each gel column, 65 mm long and 2.5 mm diameter, a constant current of 2 milliamperes was used. The resolved proteins in the gels were stained with Amido black 10B in 7.5% acetic acid for 1 hour. Excess stain was removed electrophoretically in an apparatus fabricated to the specifications of Wright and Mallmann (13). The quantities of stained proteins were estimated with an Analytrol densitometer modified to accommodate gels. A 0.9 neutral density filter was used.

Samples to be autoradiographed were labeled with ^{59}Fe. To a mixture of 25 μliters of plasma and 225 μliters of demineralized water were added 25 μliters of ^{59}Fe solution [6] (containing 0.625 μg Fe) and 25 μliters of 0.01 N HCl, which lowered the pH to 5.0. After the mixture was allowed to stand at room temperature for 20 minutes, 50 μliters of 0.01 N NaOH were added to bring the pH to 7.4. Then 405 μliters of upper gel solution were added with mixing and a 150-μliter aliquot was polymerized on each prepared gel. Following electrophoresis each gel was sliced longitudinally [7] to give two slices from the center, each of which was approximately 1 mm thick. The slice to be used for autoradiography was mounted on filter paper, which was then placed on a porous plastic block residing on a vaccum plate.[8] A sheet of thin plastic film was placed over the entire upper surface of the block, the gels were dried by a heat lamp, and reduced pressure was applied through the block. The dried gel and supporting filter paper were cut out and sandwiched tightly with two sheets of N-S (no screen) type X-ray film [9] between two glass plates. After suitable exposure the X-ray films were removed and developed. The other center slice from the same gel was stained, dried and used to identify the ^{59}Fe-labeled protein.

Total iron-building capacity (TIBC) was determined in pooled plasma samples from 7 control and 7 cadmium-fed birds by counting [10] the ^{59}Fe in the transferrin band cut from a gel immediately after comple-

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[4] In order to control dietary zinc more precisely, soybean protein diets used for studies of zinc and cadmium in our laboratory have contained sufficient nonextracted soybean protein to supply 7 mg zinc/kg diet, the level for production of moderately severe zinc deficiency in young Japanese quail by 4 weeks of age. In these studies, 7 mg zinc was supplied by 210 g of purified soybean protein RP-100 obtained from Ralston Purina Company, St. Louis, Mo. Trace-element analyses were carried out with an atomic absorption spectrophotometer, model 303, Perkin-Elmer, Norwalk, Conn.

[5] Equipment and reagents were purchased from Canal Industrial Corporation, Rockville, Md.

[6] From preliminary experiments, maximum labeling of transferrin was found to occur after 1 hour at pH 6.0, after 10 minutes at pH 5.5 and virtually instantaneously at pH 5.0. After the mixture had remained at pH 5.0 for 20 minutes, the amount of protein appearing in the transferrin band did not diminish in the plasma from either group of animals as compared to unacidified plasmas. The $^{59}Fe^{3+}$, high specific activity (15.7 mCi/mg Fe), was purchased from International Chemical and Nuclear Corporation, Pittsburgh, Pa.

[7] See footnote 5.

[8] See footnote 5.

[9] X-ray film (N-S) and photographic emulsion NTB-2 were purchased from the Eastman Kodak Company, Rochester, N. Y.

[10] Gamma Scintillation Counter, Nuclear-Chicago Corporation, Des Plaines, Ill.

tion of electrophoresis. Conditions of labeling and electrophoresis were identical with those described above. The plasma samples were digested with perchloric and nitric acids (1:5) and cleared with hydrogen peroxide. The content of iron, zinc and cadmium in the plasma was determined by atomic absorption spectrophotometry.[11]

Immunoelectrophoretic separations were carried out in agar[12] on 2.5 × 7.5 cm microscope slides by the method of Scheidegger (14). Barbital buffer,[13] with an ionic strength of 0.10 and pH 8.6, was used. The plasma proteins were separated by a constant current of 7.5 milliamperes per slide.

Whole quail plasma antiserum, produced in rabbits, was used throughout. After the removal of excess antigen and antiserum the slides were covered with perforated filter paper and dried at 40°. Dried plates were either stained with Amido black 10B and redried, or coated with photographic emulsion[14] for autoradiography. In some experiments a transverse core section of polyacrylamide gel, cut from the transferrin region immediately after the disc gel electrophoretic separation, was placed in the antigen well and subjected to immunoelectrophoresis.

RESULTS

When birds were fed cadmium at a mole ratio of 1.7:1 (zinc–cadmium), they grew more slowly and had a higher mortality than those fed the diet containing zinc alone, whereas feather scores were similar (table 1). Data on hematocrits and plasma proteins of the control and cadmium-fed birds are presented in table 2. In preliminary experiments, the plasma protein patterns were normal in nonanemic birds fed 75 mg cadmium/kg. Therefore, only the severely anemic birds fed cadmium, those whose hematocrits were 30% or less, were studied. They comprised about 80% of the cadmium-fed birds. The quantity of total plasma proteins of the severely anemic birds was similar to that of the controls. In figure 1 are photographs of plasma protein patterns obtained by disc gel electrophoresis for control and cadmium-fed birds. The amount of protein in the band designated transferrin (identification described below) increased markedly, whereas albumin and band 7 decreased in cadmium-fed birds as compared with the controls. The magnitude of these changes is shown by data in table 2 on the percentages of the plasma protein fractions determined densitometrically. All of the differences were statistically significant. As a result of changes in these fractions the transferrin–albumin ratio increased significantly in the cadmium-fed birds. Typical data for individual animals are plotted in figure 2. Although the two groups represent obviously different populations, there was no correla-

[11] See footnote 4.
[12] Ionagar No. 2, Consolidated Laboratories, Inc., Chicago Heights, Ill.
[13] Scientific Products, Washington, D. C.
[14] See footnote 9.

TABLE 1

Effect of cadmium on growth, mortality and feathering

Dietary zinc	Dietary cadmium	Time in weeks			
		1	2	3	4
mg/kg	*mg/kg*		*Body weight,[1] g*		
75	0	17.8 ± 0.48	36.0 ± 1.87	56 ± 2.5	78 ± 2.0
75	75	15.6 ± 0.69	28.0 ± 1.24	36 ± 1.3	48 ± 2.2
		$(P < 0.02)$	$(P < 0.01)$	$(P < 0.001)$	$(P < 0.001)$
			Cumulative mortality,[2] %		
75	0	—	6	8	8
75	75	—	15	31	46
			Feather score[3]		
75	0	—	57	72	80
75	75	—	69	75	79

[1] Mean values ± SE of group means from 8 experiments. A total of 65 birds fed the zinc-containing control diet and 74 fed the cadmium-containing diet were alive at 1 week.
[2] Mortality is calculated on the basis of 1-week survivors, since deaths during the first week were due to causes unrelated to the experiment.
[3] Perfect feathering is 100.

TABLE 2

Relation of plasma albumin, band 7 and transferrin concentrations to cadmium-induced anemia at 4 weeks of age [1]

Dietary zinc	Dietary cadmium	No. birds	Hematocrit	Plasma proteins	Band 7	Transferrin	Albumin	Transferrin–Albumin
mg/kg	*mg/kg*		%	*g/100 ml*	%	%	%	
75	0	30	42 ± 0.4	3.61 ± 0.112	28.6 ± 0.5	15.0 ± 0.4	30.9 ± 0.7	0.49 ± 0.018
75	75	29	22 ± 0.9	3.71 ± 0.148	23.3 ± 0.6	23.2 ± 0.5	27.1 ± 0.4	0.86 ± 0.025
			—	($P < 0.2$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)	($P < 0.001$)

[1] Data on cadmium-fed birds are from those with hematocrits below 30%; this degree of anemia occurred in approximately 80% of these birds. Mean values ± SE.

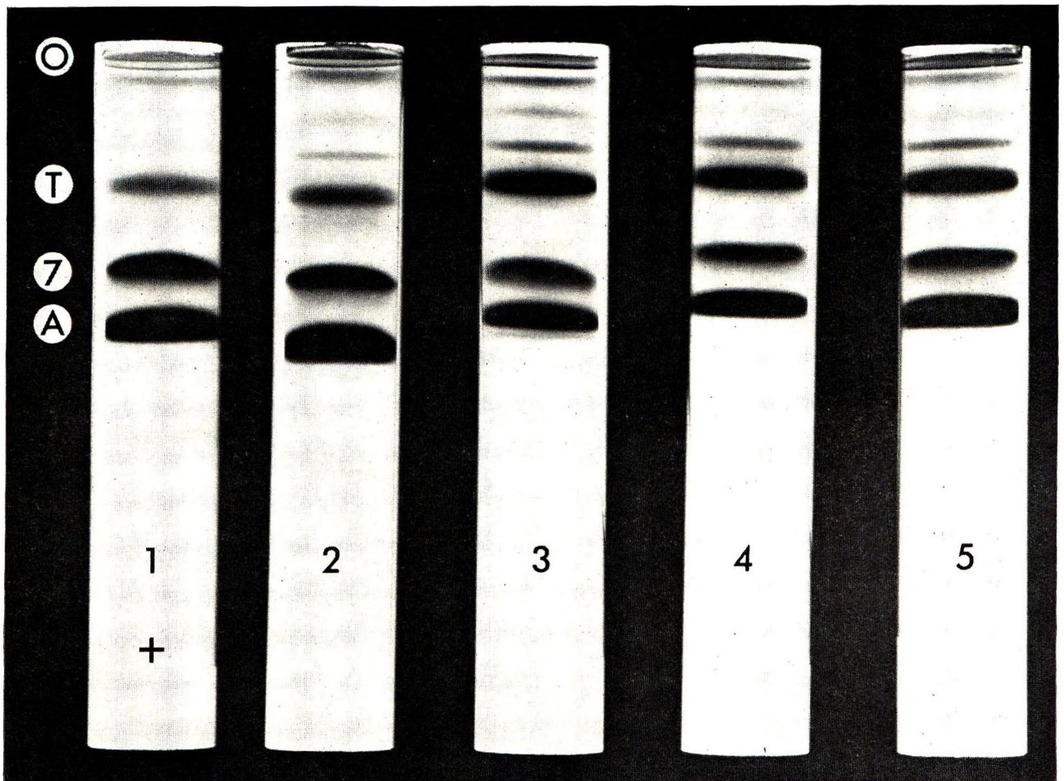

Fig. 1 Pattern of plasma proteins from control birds (1, 2) and cadmium-fed birds (3–5), separated by disc electrophoresis. Bands are identified: origin, 0; transferrin, T; band 7, 7; albumin, A. Migration was toward the anode, +. All gels had two bands at the origin; "appearance" of more than two bands for gels 1 and 2 is a photographic artifact. Each gel contained 3 μliters of plasma and was stained with Amido black 10B.

tion between hematocrit and ratio of transferrin to albumin within groups.

The identity of the band referred to as transferrin is more completely shown in figure 3. The bulk of the ^{59}Fe was not bound to protein and migrated in the region of the leading chloride boundary. The bound ^{59}Fe was present in the exact location of the protein-staining band that migrated behind band 7. The increased protein-staining of this band in plasma from cadmium-fed birds was also accompanied by an increase in ^{59}Fe concentration. The plasma iron content was 277 and 100 μg/100 ml for control and cadmium-fed birds, whereas the TIBC was 186 and 377 μg iron/100 ml,

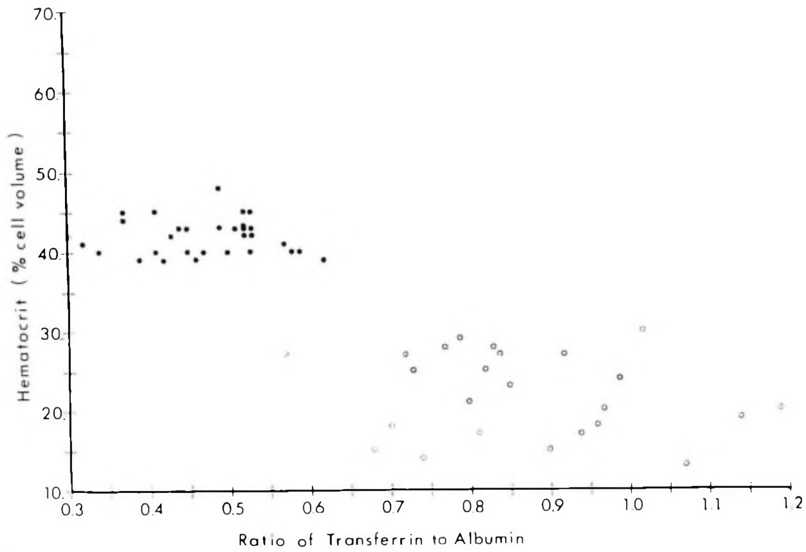

Fig. 2 Relationship of hematocrits to transferrin–albumin in control and cadmium-fed birds at 4 weeks of age. Dietary zinc and cadmium (mg/kg): ● 75 zinc; ○ 75 zinc and 75 cadmium.

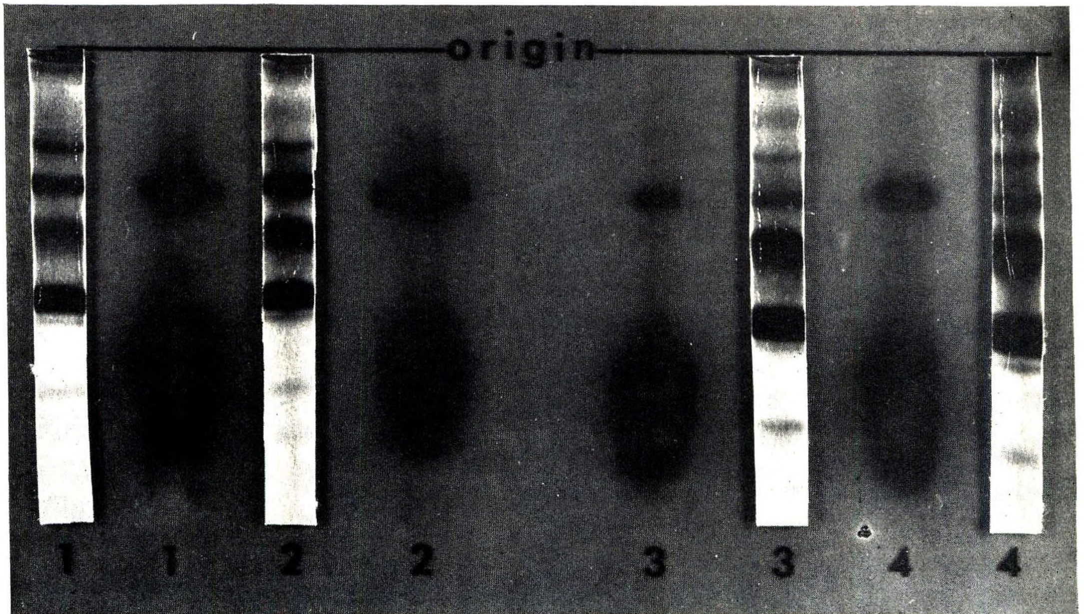

Fig. 3 Autoradiographs and stained disc gel slices of plasma labeled in vitro with ^{59}Fe. See figure 1 for identification of bands. Patterns 1 and 2 are from cadmium-fed birds, 3 and 4 from controls. Each gel contained 5 μliters of plasma and identical amounts of ^{59}Fe.

respectively. The concentration of zinc in the plasma was 299 and 152 μg/100 ml and the concentration of cadmium was 20 and 24 μg/100 ml plasma from control and cadmium-fed birds, respectively.

Immunoelectrophoresis of whole quail plasma and transverse core sections from the transferrin area of disc gels are shown in figure 4. The immunoelectrophoretic patterns are identical, as would be expected. The precipitin arc obtained with the transferrin core section compared favorably with the arc obtained with whole plasma from both control and cadmium-fed birds. Most important is the singularity of these bands, indicating that only one normal protein was migrating in this area. Transferrin is again identified with respect to its iron-binding property, as shown in the accompanying autoradiographs of figure 4.

In figure 5 the relative amounts of transferrin present in the cadmium-fed birds, as compared with controls, are shown. Upon graded dilution, the transferrin precipitin band disappeared more rapidly from the plasma pattern of the control bird than did that in the pattern from the cadmium-fed bird, indicating increased transferrin levels in the latter plasma.

DISCUSSION

The general antagonism of zinc by cadmium has long been recognized; however, the physiological consequences, particularly as related to fine quantitative adjustments between the two elements, and the mechanism of action in the diversity of affected biological systems is largely unknown. In these studies a level of cadmium was chosen to reduce the growth rate of the young Japanese quail at 4 weeks of age to that of the severely zinc-deficient quail (7). The zinc–cadmium mole ratio of 1.7:1 was generally satisfactory at a dietary level of 75 mg/kg diet. Greater relative amounts of cadmium caused excessive mortality, whereas lesser amounts produced considerably less severe effects on mortality and growth. The level of 75 mg/kg of diet is more than twice the

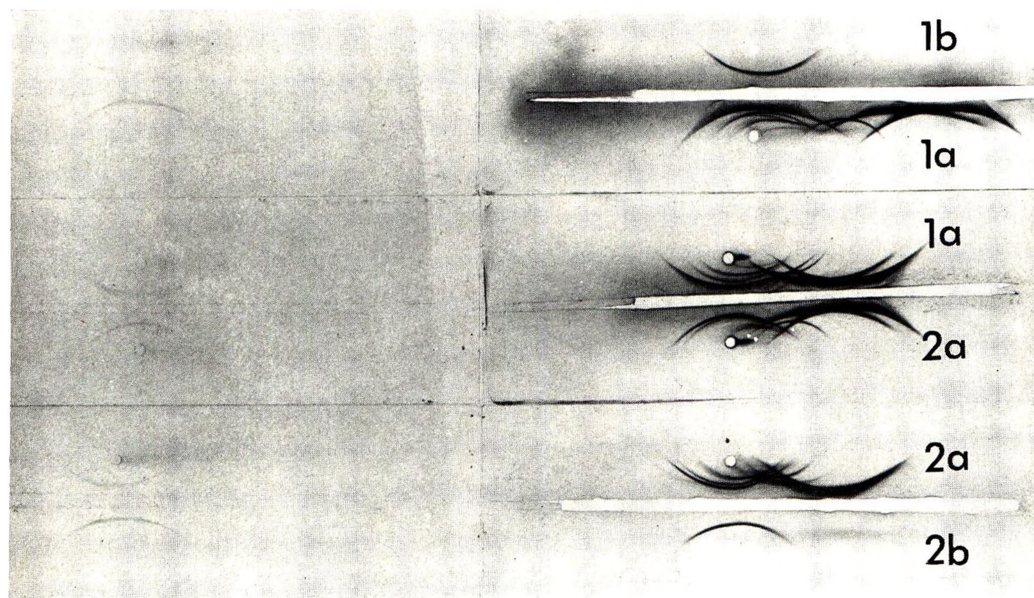

Fig. 4 Immunoelectrophoresis and autoradiography (left) of whole plasma and transferrin previously separated by disc gel electrophoresis. Patterns labeled 1 were obtained with plasma from a cadmium-fed bird; those labeled 2 were from a control. All "a" patterns resulted from separation of 3 μliters of whole plasma; all "b" patterns were from a 2 mm gel core punched from the unstained transferrin area of a disc gel immediately after separation of 5 μliters of plasma. All troughs contained 75 μliters of whole quail antiserum.

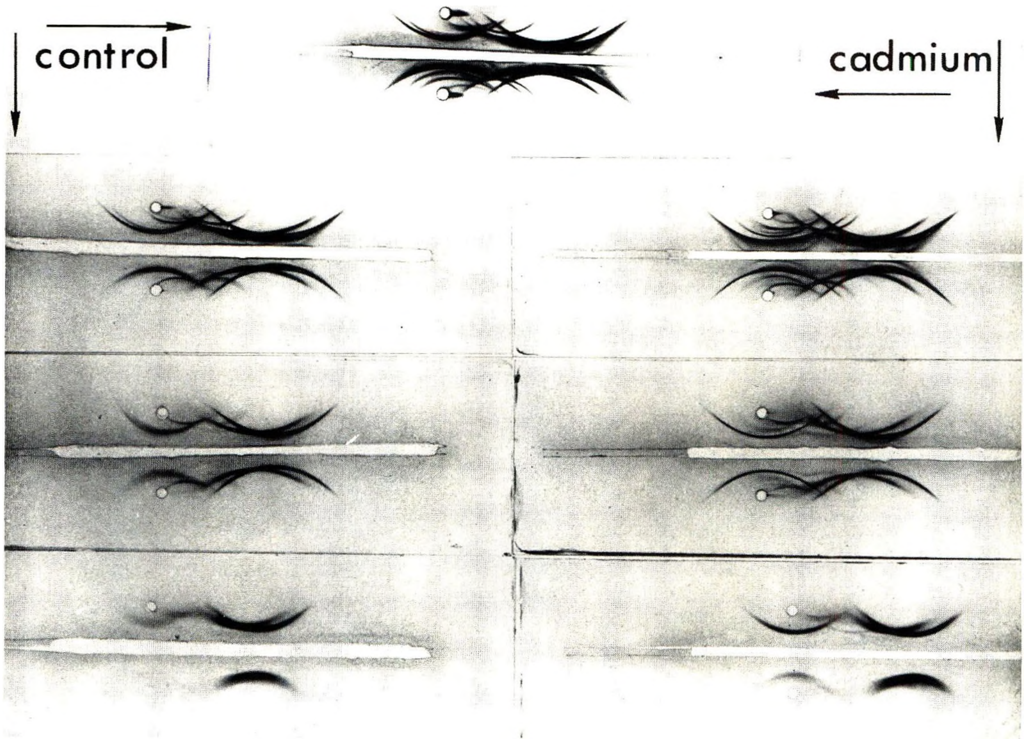

Fig. 5 Immunoelectrophoretic patterns with graded dilutions (3, 2, 1, 0.75, 0.5, 0.25, and 0.1 μliter/well) of plasma from control and cadmium-fed birds. All center troughs contained 75 μliters of whole quail plasma antiserum. For identification of the transferrin arc, see figure 4.

zinc requirement for the young Japanese quail.[15]

In addition to poor growth, other parallelisms between zinc deficiency and cadmium toxicity were apparent. These included very small testes [16] and lower concentrations of the principal plasma lipoprotein, band 7, in cadmium-fed birds and in fasted zinc-deficient birds (8, 9). The latter effect may reflect cardiovascular difficulties such as have been reported in other species receiving cadmium (1, 2). Some of the major adverse effects of zinc deficiency such as poor feathering and deformities of the long bones [17] were not mimicked by cadmium toxicity. The marked effects of cadmium in precipitating anemia, decreased plasma band 7, and elevated plasma transferrin levels are not characteristic of zinc-deficient quail at 4 weeks of age. It appears likely that the anemia and elevation of transferrin levels were due to interference with iron absorption or metabolism or both.

The disc gel electrophoresis of plasma from anemic cadmium-treated birds did not show an abnormal serum component (ASC), as Lawford (15, 16) found in plasma from rats. The production of ASC by cadmium was elicited only in male rats with normally functional testes (17).

Renal damage and proteinuria from exposure to cadmium has been described in man and in laboratory animals (18–22). Albumin (18) was identified as one of the urinary proteins together with larger amounts of protein(s) with a lower molecular weight, in the 20,000 to 30,000 range. Kench et al. (23, 24) designated these as minialbumins because of the vari-

[15] Fox, M. R. S., and R. M. Jacobs 1967 Zinc requirement of the young Japanese quail. Federation Proc., 26: 524 (abstract).

[16] Unpublished data.

[17] See footnotes 15 and 16.

ous physical-chemical and immunochemical similarities to albumin. More recently, plasma albumin of low tryptophan content and tryptophan-free urinary minialbumins were found in cadmium-treated monkeys (25). From these and other observations, Kench et al. concluded that the minialbumins were derived from albumin. Although we have not investigated urinary excretion of albumin or minialbumins by the quail, we have observed a lower plasma concentration of albumin in the cadmium-treated bird together with kidney damage.[18] Whether the decreases in plasma albumin concentration and the minialbumin proteinuria are due entirely to kidney damage or are actually a manifestation of abnormal albumin synthesis or catabolism remains to be established.

The specificity of the plasma transferrin increase in response to dietary cadmium is reasonably certain in these experiments. More than one protein can migrate at a given rate under conditions of any one type of electrophoresis; however, the use of two completely different types of electrophoresis, the specificity of immunochemical techniques and the increases in TIBC and in ^{59}Fe-binding by proteins in the transferrin regions all support an interpretation of elevation of transferrin per se.

The time of onset and the characteristics of the anemia and the elevation of plasma transferrin in cadmium-fed Japanese quail need further study. However, the adverse hematological effects of cadmium seem to be closely related to interference with iron metabolism. Rabbits fed cadmium developed hyperplastic bone marrow and hypochromic microcytic anemia, similar to that produced by iron deficiency (26, 27). There was evidence of increased phagocytosis of red blood cells and enlarged spleens; the red blood cells became more sensitive to changes in osmotic pressure (27). In rabbits made anemic by cadmium, parenteral administration of iron corrected the anemia (27).

Several mechanisms may be postulated to explain the effect of cadmium in elevating the plasma level of transferrin in the Japanese quail. Transferrin functions as the iron donor in the formation of heme (28). During this process it is bound to reticulocytes (29–31) and reticuloendothelial cells (32). Jandl and Katz (30) suggested that transferrin is partitioned between the extra- and intravascular pools by virtue if its iron saturation, with the greater saturation in the former. In the rat the transferrin partition ratio, extravascular–intravascular, was shown to be in the range of 1.5 to 1.7 (33–35). Cadmium-poisoned rabbits have been shown to have iron-deficient marrow (27). Since quail synthesize the majority of their hemoglobin intravascularly, the quail with cadmium-induced iron deficiency would be expected to exhibit plasma hypertransferrinemia due to the decreased binding of transferrin to erythrocytes in the absence of adequate iron. Knowledge of the distribution and catabolism of transferrin in the quail would be necessary to support such an hypothesis. On the other hand, Morgan and others (36–38) have shown that transferrin in the rat and rabbit is affected by two mechanisms: one, specific for transferrin, depends on the oxygen supply; the other represents a nonspecific response to disturbances of plasma protein metabolism. Rosse and Waldmann (39) have shown that Japanese quail are sensitive to a humoral erythropoietic substance; the release of this substance was regulated by the oxygen supply. Since our birds were severely anemic, had enlarged hearts [19] and most certainly were hypoxic, the humorally regulated mechanism could probably explain the maintenance of high levels of plasma transferrin. In the case of rats made anemic by phenylhydrazine, the elevated plasma level of transferrin was shown by Morgan (33) to be due to increased synthesis and decreased catabolism of transferrin and to its movement from the extravascular to the intravascular space. The predicted erythropoietin increases coupled with a high red cell destruction rate and ineffectual erythropoiesis no doubt would keep the bird iron-deficient and anemic. A lack of iron would allow the transferrin to be released to the plasma and the stimulated erythropoietic mechanism would reduce the catabolism of transferrin, giving rise to plasma hypertransferrinemia.

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[18] See footnote 16.
[19] See footnote 16.

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Vitamin A Activity of Corn Carotenes for Swine [1]

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ABSTRACT Three trials, using 204 weanling pigs, were conducted to estimate the vitamin A potency of corn carotenes for the pig. The pigs were depleted of their vitamin A stores by feeding a wheat- or milo-based diet until serum vitamin A levels approached or fell below 10 μg/100 ml. After depletion the pigs were assigned at random to one of the following seven repletion diets: 1) basal diet; 2) basal diet supplemented with 3 levels of carotene from corn or corn gluten meal, or both, with carotene concentrations from 1.04 to 10.3 mg/kg of diet; 3) basal diet supplemented with 3 levels of all-*trans*-retinyl palmitate, with retinyl palmitate concentrations from 73 to 654 μg/kg diet. Repletion diets were fed until the pigs reached market weight. Serum vitamin A determinations were made initially and periodically throughout the trials. Liver vitamin A stores were monitored by collecting livers from three or four pigs from each of the three trials at the start and end of depletion, and from all pigs at the end of repletion. The carotenes in corn and corn gluten meal were separated by column chromatography, identified as to structural and stereoisomeric form, and quantitated spectrophotometrically. Estimates of vitamin A potency were based on total liver vitamin A stores which the carotenes supported during repletion. Estimating equations were calculated by regressing the logarithm of total vitamin stores in milligrams/liver on total milligrams of carotene or retinyl palmitate consumed during repletion. Conversion efficiencies were calculated by dividing the regression coefficients for carotene by the regression coefficients for retinyl palmitate. At higher levels of carotene intake, when corn gluten meal was included in the repletion diets, 1 mg of carotene had a vitamin A potency of 123 to 174 IU. When corn was the only source of carotenes and the concentration was more typical of corn-soy swine diets, 1 mg of carotene had a vitamin A potency of 261 IU. It would appear that the NRC relationship, between 1 mg of dietary carotene and 500 IU of vitamin A activity, exaggerates the usefulness of corn carotenes for swine when liver vitamin A storage is used as the criterion.

The Subcommittee on Swine Nutrition of the National Academy of Sciences–National Research Council (1) has set the carotene requirement of the growing-finishing pig at 2.6 mg/kg diet. This figure was based on "1 mg of β-carotene equaling 500 IU of biologically active vitamin A."

Because most natural feeds contain variable amounts of isomers other than all-*trans*-β-carotene (usually considered most biologically active), it is likely that the vitamin A activity of feed carotenes for swine is not a constant but varies with a number of factors, including the structural and stereoisomers that are present.

Even when dietary carotenes are essentially all-*trans*-β-carotene, their biopotency for swine may not be as great as suggested by the NAS–NRC (1). Ullrey et al. (2) studied a preparation produced in a fermentation system by the heterothallic mold, *Blakeslea trispora*, which was 94-96% all-*trans*-β-carotene by assay. When vitamin A storage in the liver was used as the primary criterion, 1 mg of this fermentation carotene was equivalent to 105.6 μg (192 IU) of dietary all-*trans*-retinyl palmitate for the vitamin A-depleted pig of 50 to 100 kg body weight. Hendricks et al. (3), using this same fermentation carotene, found that 1 mg was equivalent to 83.6 μg (152 IU) of all-*trans*-retinyl palmitate for the 10-kg pig.

Madsen and Earle (4), using the rat, noted that the carotenes of yellow corn had a biopotency of 762 IU/mg, which is 45.7% of the theoretical value of 1667 IU/mg of all-*trans*-β-carotene. Isomers other than all-*trans*-β-carotene are known to be present in yellow corn, and Sadana and Ahmad (5) used analytical values to calculate expected biopotencies for corn

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carotenes. They assumed that 1 IU of vitamin A activity could be derived from 0.6 μg of all-*trans*-β-carotene or from 1.2 μg of other provitamin A carotenes which were present. Based on these factors and the concentrations of the various carotenes, they predicted that 1 mg of corn carotenes would have a biopotency of 1041 IU for the rat.

Because yellow corn is a major feed for swine in many parts of the United States, and it is the primary carotene source in these feeds, an understanding of the vitamin A potency of corn carotenes would provide a basis for more precise swine feed formulation. Our previous experience with swine (2, 3) suggested that estimates of carotene biopotency for this species, based on research with rats, were likely to be incorrect. It seemed appropriate, therefore, to design and conduct the following experiments.

MATERIALS AND METHODS

A total of 204 weanling pigs were used in three trials. The general plan was to take the pigs shortly after weaning, when they were 5 to 7 weeks old, and assign them to the vitamin A depletion diet shown in table 1. In trials 1 and 3, their mothers had been fed a vitamin A-low diet from just prior to parturition throughout lactation. In trial 2, their mothers had received a standard, vitamin A-containing diet during gestation and lactation. The creep feed provided in all trials was low in vitamin A.

TABLE 1

Composition of basal diet

	%
Ground wheat (or milo) [1]	86.2
Soybean meal, 49% crude protein	11.0
Dicalcium phosphate, 26% Ca, 18% P	1.0
Limestone, 38% Ca	0.9
Trace mineral salt [2]	0.5
B-vitamin supplement [3]	0.1
B_{12} supplement, 13 mg/kg	0.1
Lysine supplement, 50% L-lysine [4]	0.07
DL-Methionine [5]	0.02
Zinc oxide	0.005
Antibiotic supplement [6]	0.1
Vitamin D_2 supplement, 9000 IU/kg	2.6 g/kg

[1] Milo used in trial 1, only; ground wheat or milo were replaced by appropriate amounts of corn, or corn and corn gluten meal, or a wheat-based retinyl palmitate premix, for the repletion diets.

[2] Contains not less than: (in percent) NaCl, 97; Mn, 1.2; Zn, 0.8; Fe, 0.33; Cu, 0.05; Co, 0.02; I, 0.01 (Michigan Salt Co., St. Louis, Michigan).

[3] Four milligrams riboflavin, 9 mg pantothenic acid, 20 mg nicotinic acid and 200 mg choline chloride/kg (Dawe's Laboratories, Inc., Chicago).

[4] Lyamine. Supplied through the courtesy of L. Michaud, Merck and Co., Rahway, New Jersey.

[5] Supplied through the courtesy of T. A. Hymas, Dow Chemical Co., Midland, Michigan.

[6] Eleven grams procaine penicillin and 33 g streptomycin sulfate/kg (Merck and Co., Rahway, New Jersey).

Blood samples were taken from the anterior vena cava (6) initially and at 3-week intervals until blood serum vitamin A concentrations approached or fell below 10 μg/100 ml. Either three or four pigs were killed at the beginning and at the end of depletion to establish liver vitamin A levels.

The pigs were then allotted at random from breed, sex and weight outcome groups to one of the seven treatments shown in table 2. Blood samples were taken at 3 and 6 weeks and at the end of the experimental period. The pigs were killed and the livers were removed, weighed and a sample was taken from the distal one-half of the left central lobe of each liver for vitamin A analysis.

Repletion diets. The repletion diets were developed by modifying the depletion diet as illustrated in table 2. Ground wheat or milo was replaced by corn, or corn plus corn gluten meal,[2] to provide three dietary levels of carotene from corn. Likewise a ground wheat- (or milo-) based premix containing retinyl palmitate was substituted for ground wheat or milo in the depletion diet to provide three dietary levels of preformed vitamin A activity. The retinyl palmitate was obtained commercially as a vitamin A feed supplement. It contained 192.1 mg of all-*trans*-retinyl palmitate/gram (by assay) plus edible tallow, gelatin, lactose and 2% of ethoxyquin. Particle surfaces were treated with sodium silico aluminate.[3]

The diets were mixed approximately every 10 days in trials 1 and 3, and just once in trial 2. Samples for carotene assay were taken at the time of mixing and when the last of the batch was fed in trials 1 and 3. Samples were taken for carotene assay at the beginning, middle and end of repletion in trial 2. The feed

[2] "Prairie Gold" 60% (CP) corn gluten meal. Supplied through the courtesy of Victor Heiman, Corn Products Sales Co., New York.

[3] PGB-325S Dry Vitamin A Feed Supplement. Supplied through the courtesy of S. R. Ames, Director, Biochemical Research Laboratories, Distillation Products Industries, Rochester, New York.

TABLE 2

Crude carotene and retinyl palmitate concentration of repletion diets

Lot	Treatment	Concentration		
		Trial 1	Trial 2	Trial 3
			mg/kg	
1	Basal diet	0	0	0
2	Basal diet + corn or corn and corn gluten meal carotenes	1.04 ± 0.11 [1]	1.46 ± 0.09	1.05 ± 0.05
3	Basal diet + corn or corn and corn gluten meal carotenes	2.77 ± 0.11	4.08 ± 0.20	1.81 ± 0.24
4	Basal diet + corn or corn and corn gluten meal carotenes	10.20 ± 0.32	10.30 ± 0.42	5.52 ± 0.17
			μg/kg [2]	
5	Basal diet + retinyl palmitate	73	73	73
6	Basal diet + retinyl palmitate	218	218	218
7	Basal diet + retinyl palmitate	654	654	654

[1] SE of crude carotene analyses.
[2] As calculated from manufacturer's assay.

was mixed in a darkened room and stored in cloth sacks in a darkened area.

Diet assays. Carotene isomer separation and quantitation were carried out according to the method of Quackenbush et al. (7). Crude carotene concentrations were determined by modifying the isomer separation so that fractions 1, 2 and 3, which contained all of the provitamin A active carotenes in corn, were eluted together using a developing solution consisting of 90% hexane [4] and 10% acetone. The eluant was appropriately diluted with hexane, and the optical density of the resulting mixture was determined at 440 mμ in a Beckman DU spectrophotometer and compared with a standard curve developed using a 100% β-carotene standard.[5] These produced carotene values comparable to those resulting from the AOAC (8) procedure.

Dietary retinyl palmitate concentrations were not determined directly but were calculated on the basis of analyses of the retinyl palmitate premix performed by the supplier. This plan was followed because of the relatively poor accuracy and precision of vitamin A analyses at such low concentrations in feeds. Satisfactory homogeneity of the feed and mixing efficiency was established by a series of sample analyses of proximate composition and of concentrations of calcium, phosphorus and magnesium.

Serum and liver vitamin A assay. Serum and liver samples were stored at −25° until analyses could be performed. Serum vitamin A concentrations were determined using the antimony trichloride method of Embree et al. (9). Liver vitamin A concentrations were determined according to Embree et al. (9) with modifications as described by Martin et al. (10). Both tissues were also checked for carotene concentration.

Statistical analyses. The data were examined for statistical significance by least squares analysis. Mean differences were compared using Duncan's multiple range test (11).

RESULTS AND DISCUSSION

Dietary assays. Results of the crude carotene assays are shown in table 2. Each carotene value represents the average of analyses on all samples taken, whether at the time of mixing or at a later date, as there were no significant differences and thus no detectable losses in crude carotene concentration during storage in any of the 3 trials. There was no detectable carotene in the depletion diet.

The concentrations of the carotene isomers in the corn and corn gluten meal used in these trials are shown in table 3.

[4] Skellysolve B, Skelly Oil Co., Kansas City, Missouri.
[5] 100% β-carotene, Catalog No. 3702, Eastman Organic Chemicals Dept., Distillation Products Industries, Rochester, New York.

TABLE 3

Carotenes in corn and corn gluten meal

Component	Corn 1 [1]	Corn 2 [1]	Corn 3 [1]	CGM1 [2]	CGM2 [2]
			mg/kg		
Phytoene	3.7 ± 0.4 [3]	5.0 ± 0.2	8.7 ± 0.6	64.7 ± 2.4	53.2 ± 1.5
Phytofluene	2.0 ± 0.1	6.1 ± 1.4	4.0 ± 0.2	69.7 ± 4.6	44.0 ± 1.6
β-Carotene	0.5 ± 0.0	1.3 ± 0.0	2.0 ± 0.0	13.8 ± 0.4	20.1 ± 0.1
β-Zeacarotene	0.4 ± 0.0	1.2 ± 0.0	1.9 ± 0.0	12.6 ± 0.4	20.9 ± 0.1
Zeta-carotene	0.2 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	7.4 ± 0.1	13.7 ± 0.1
Zeinoxanthin	0.9 ± 0.1	2.1 ± 0.1	4.0 ± 0.1	42.5 ± 4.0	40.5 ± 1.4
Cryptoxanthin	0.9 ± 0.1	1.9 ± 0.1	3.7 ± 0.1	35.6 ± 2.6	36.3 ± 0.8
Esters	2.5 ± 0.1	11.8 ± 2.1	16.0 ± 0.3	81.2 ± 3.5	176.9 ± 1.0
Lutein	2.6 ± 0.1	12.2 ± 2.1	16.2 ± 0.2	84.5 ± 3.4	184.8 ± 0.3
Zeaxanthin	0.7 ± 0.0	4.2 ± 0.8	6.5 ± 0.2	31.5 ± 1.2	80.7 ± 0.2
Polyoxy pigments	0.9 ± 0.0	1.9 ± 0.0	1.4 ± 0.1	7.7 ± 0.2	22.4 ± 0.9
Total	15.3	48.4	65.5	451.2	693.5

[1] Corn used in trials 1, 2 and 3, respectively.
[2] Corn gluten meal used in trials 1 and 2, respectively.
[3] SE of analyses.

TABLE 4

Provitamin A-active components of corn and corn gluten meal

Component	Corn 1 [1]	Corn 2 [1]	Corn 3 [1]	CGM1 [2]	CGM2 [2]
			% active pigments		
β-Carotene	27.8	29.5	26.3	22.3	26.0
β-Zeacarotene	22.2	27.3	25.0	20.3	27.0
Cryptoxanthin	50.0	43.2	48.7	57.4	47.0
			mg/kg		
Total active	1.8	4.4	7.6	62.0	77.3
Crude carotene	2.1 ± 0.1 [3]	5.1 ± 0.2	8.6 ± 0.1	69.7 ± 1.7	90.8 ± 1.4

[1] Corn used in trials 1, 2 and 3, respectively.
[2] Corn gluten meal used in trials 1 and 2, respectively.
[3] SE of analyses.

The concentrations of the provitamin A-active isomers are presented in table 4 as percentages of the total active pigments. These percentages were relatively constant from sample to sample, but the total active carotene concentrations varied considerably. Table 4 also presents the opportunity to compare the total active carotene concentrations, obtained by summing the concentrations of the individual active pigments, with the results of the crude carotene analyses. The figures are similar except at high pigment concentrations where the correction factors (7) used in calculation of the individual isomer concentrations have a signficant effect in removing the interferences and interactions of the various pigments. These were not accounted for in the crude carotene determination where just one optical density reading at 440 mμ was made.

Weight gain and feed use. Daily gains during depletion were normal in all 3 trials. During repletion in trial 1, all lots of pigs receiving corn carotenes or retinyl palmitate gained significantly ($P < 0.01$) faster than the basal lot. There was a tendency for gains to increase with increasing supplemental levels, but significant ($P < 0.01$) differences were found only between the two higher levels of carotene supplementation and the lower level of carotene or retinyl palmitate supplementation.

In trial 2, two pigs grew poorly on the highest level of corn carotenes and, as a consequence, the average daily gain of the pigs in this lot was significantly ($P < 0.01$) less than that of pigs receiving the highest supplement of retinyl palmitate. There were no lesions found at slaughter which would explain this poor performance. No other signficant differences in gain between treatments were observed.

In trial 3, all supplemented lots gained significantly ($P < 0.01$) faster than the basal lot, except the lot receiving the lowest

level of retinyl palmitate. Pigs receiving the middle level of corn carotenes and those receiving the highest level of retinyl palmitate gained significantly ($P < 0.01$) faster than pigs receiving the lowest level of corn carotenes and pigs receiving the middle and lowest levels of retinyl palmitate. Pigs receiving the highest level of corn carotenes gained significantly ($P < 0.01$) faster than those receiving the lowest level of retinyl palmitate.

Serum vitamin A values. Table 5 shows the effects of depletion and repletion upon serum vitamin A concentration. No carotenes were detected in any of the samples. In trials 1 and 3, average serum vitamin A concentrations dropped below 10 μg/100 ml by the end of depletion. This desired level was not reached in trial 2.

There were no significant differences between lots in initial repletion vitamin A levels in any of the trials. However, in trial 1, castrated males had significantly ($P < 0.01$) lower serum vitamin A values (1.9 μg/100 ml less) than females.

All supplemental levels of carotenes or retinyl palmitate, except for the lowest level of retinyl palmitate in trial 2, significantly ($P < 0.01$) increased serum vitamin A concentration over the basal diet in all trials. Increasing supplemental levels tended to increase serum vitamin A concentration, and the significant differences are designated in table 5.

Liver vitamin A values. The effects of depletion and repletion upon liver vitamin A values are shown in table 6. No carotenes were detected. In trials 1 and 3, vitamin A depletion was essentially complete as measured by the liver stores of pigs killed at the end of the depletion period. In trial 2 this was not the case.

Liver vitamin A values at the end of repletion are presented in three different ways: micrograms vitamin A/gram wet liver, micrograms vitamin A/gram dry liver, and total liver vitamin A in milligrams. When treatment means were compared statistically, all three methods resulted in the same conclusions. Therefore, only the statistical analysis of total liver vitamin A in milligrams will be discussed.

In trials 1 and 3 the highest level of carotene and retinyl palmitate supplementation produced significantly ($P < 0.01$) greater total liver vitamin A than all other treatments. In trial 2 the highest level of carotene and retinyl palmitate supplementation gave significantly ($P < 0.01$) greater total liver vitamin A than the two lowest levels of retinyl palmitate and the basal diet, but were not significantly different from the two lowest levels of carotene supplementation. Pigs in all 3 trials showed

TABLE 5

Serum vitamin A concentration of pigs fed a vitamin A depletion diet and then repleted with diets containing corn or corn and corn gluten meal carotenes or retinyl palmitate

	Depletion							
Trial		1		2		3		
Length of depletion, days		56		69		71		
				μg/100 ml				
Initial value		29 ± 0.6(70) [1]		39 ± 1.7(42)		28 ± 0.8(70)		
Final value		7 ± 0.7(70)		15 ± 1.0(42)		8 ± 0.5(70)		
	Repletion							
Lot	1	2	3	4	5	6	7	
Diet	B [2]	LC	MC	HC	LA	MA	HA	
Final value				μg/100 ml [3]				SE
Trial 1, 63 days	5(5) [a]	14(10) [b]	18(10) [b,c]	33(10) [d]	16(10) [b]	22(10) [c]	32(10) [d]	1.6
Trial 2, 41 days	6(6) [b]	14(6) [b]	20(6) [c]	20(6) [c]	4(6) [a]	14(6) [b]	24(5) [d]	1.3
Trial 3, 61 days	4(7) [a]	9(7) [c]	11(9) [d]	15(10) [e]	6(6) [b]	10(9) [c,d]	21(10) [f]	0.8

[1] Mean ± SE with the number of pigs in parentheses.
[2] B = basal; LC, MC and HC = low, medium and high carotene, respectively; LA, MA and HA = low, medium and high vitamin A, respectively.
[3] Number of pigs in parentheses. Values in same trial with different superscripts are significantly different ($P < 0.01$).

TABLE 6

Liver vitamin A concentration of pigs fed a vitamin A depletion diet and then repleted with diets containing corn or corn and corn gluten meal carotenes or retinyl palmitate

				Depletion				
Trial			1		2		3	
Length of depletion, days			56		69		71	
Initial value								
Conc (dry), μg/g			51.2±9.0(4) [1]		74.2±7.5(3)		24.8±6.0(4)	
Total, mg/liver			5.5±0.8(4)		17.3±1.5(3)		2.8±0.2(4)	
Final value								
Conc (dry), μg/g			2.3±0.4(4)		10.0±3.9(3)		0.2±0.1(4)	
Total, mg/liver			0.5±0.1(4)		4.8±2.1(3)		0.1±0.0(4)	
				Repletion				
Lot	1	2	3	4	5	6	7	
Diet	B [2]	LC	MC	HC	LA	MA	HA	
Trial 1, 63 days								SE
Conc (wet), μg/g	0.6 [a]	0.5 [a]	0.8 [a]	7.0 [b]	0.6 [a]	1.1 [a]	6.9 [b]	0.35
Conc (dry), μg/g	2.4 [a]	1.8 [a]	2.7 [a]	23.7 [b]	2.2 [a]	3.9 [a]	23.2 [b]	1.19
Total, mg/liver	0.8 [a]	0.7 [a]	1.2 [a]	10.0 [b]	0.9 [a]	1.6 [a]	9.8 [b]	0.54
Trial 2, 41days								
Conc (wet), μg/g	0.4 [a]	1.7 [a,b]	2.4 [b]	2.4 [b]	0.4 [a]	0.9 [a]	2.4 [b]	0.19
Conc (dry), μg/g	1.4 [a]	5.8 [a,b]	8.3 [b]	7.7 [b]	1.5 [a]	3.0 [a]	7.9 [b]	0.62
Total, mg/liver	0.7 [a]	2.7 [a,b,c]	3.6 [b,c]	4.3 [c]	0.8 [a]	1.5 [a,b]	4.8 [c]	0.65
Trial 3, 61 days								
Conc (wet), μg/g	0.2 [a]	0.2 [a]	0.9 [a]	2.6 [b]	0.3 [a]	0.5 [a]	2.6 [b]	0.14
Conc (dry), μg/g	0.7 [a]	0.6 [a]	3.1 [a]	8.8 [b]	0.9 [a]	1.7 [a]	8.5 [b]	0.46
Total, mg/liver	0.2 [a]	0.2 [a]	1.1 [a]	3.0 [b]	0.3 [a]	0.7 [a]	3.7 [b]	0.25

[1] Mean ± SE with the number of pigs in parentheses.
[2] B = basal; LC, MC and HC = low, medium and high carotene, respectively; LA, MA and HA = low, medium and high vitamin A, respectively. Values on same line with different superscripts are significantly different ($P < 0.01$).

a progressive increase in mean total liver vitamin A from the lowest to the highest level of supplementation for both carotene and retinyl palmitate.

Calculation of vitamin A potency of corn carotenes. The vitamin A activity of the corn carotenes was compared with all-*trans*-retinyl palmitate by calculating the regression of the logarithm of total liver vitamin A storage during repletion upon total dietary vitamin A intake in milligrams (12). By dividing the regression coefficient of the carotene source by the regression coefficient of the reference material (all-*trans*-retinyl palmitate) and multiplying by 100, the potency of the carotene source as a percentage of the reference was determined. By multiplying the theoretical vitamin A activity of all-*trans*-retinyl palmitate (1818 IU/mg) by this percentage biopotency of carotenes, an IU value for 1 mg of crude carotenes as found in the corn, or corn and corn gluten meal, was calculated.

The regression equations fitted to the data by the method of least squares are as follows (Y = total mg vitamin A stored per liver, X_A = total mg of all-*trans*-retinyl palmitate ingested during repletion, and X_C = total mg of crude carotene ingested during repletion):

Trial 1 $\log Y = -0.2656 + 0.01030X_A + 0.0006960X_C$

Trial 2 $\log Y = -0.004275 + 0.007773X_A + 0.0007430X_C$

Trial 3 $\log Y = -1.0299 + 0.01331X_A + 0.001907X_C$

The relative potencies of the carotenes in corn, or corn and corn gluten meal, as compared with all-*trans*-retinyl palmitate, are shown in table 7. The values of 123 and 174 IU/mg derived from trials 1 and 2, respectively, are quite similar considering that the pigs used in trial 2 were rather incompletely depleted. Both corn and corn

TABLE 7

Vitamin A activity of corn carotenes and efficiency of conversion by the pig

Trial	Conversion efficiency, % [1]	IU/mg [2]
1	6.76	123
2	9.56	174
3	14.33	261

[1] Calculated by dividing the regression coefficient of the carotene source by the regression coefficient of the all-*trans*-retinyl palmitate and multiplying by 100.
[2] Calculated by multiplying the vitamin A activity of all-*trans*-retinyl palmitate (1818 IU/mg) by the relative efficiency of conversion of crude corn carotenes.

gluten meal were included in the diets used in these trials. In trial 3, where only corn was used, the relative potency of the carotenes was 261 IU/mg. Since the proportion of vitamin A-active pigments was essentially the same in corn and corn gluten meal, it does not seem likely that the difference in the relative concentration of the carotene isomers in the diets provides the explanation for the differing potency estimates. Of course, availability of the isomers in corn gluten meal could conceivably have been less than that of the isomers in corn.

There was a rather large difference, however, between the total concentration of crude carotenes in the diets used in trial 3 as compared to the concentration in the diets used in trials 1 and 2. The greatest difference was between the high carotene diets in the three trials, and this concentration in trial 3 was only 54% of that in trials 1 and 2. Myers et al. (13) have observed that the efficiency of conversion of alfalfa carotenes to vitamin A by the pig was inversely related to dietary carotene concentration, and this inverse relationship may have influenced our findings as well. At the end of depletion the pigs in trial 3 were also more completely depleted, and, thus, were perhaps more penurious with the vitamin A activity in the repletion diets.

A calculation of the theoretical biopotencies of the "active" carotene pigments in corn and corn gluten meal produces some interesting figures for comparison with the potencies determined experimentally. If one arbitrarily assigns a relative potency of 100 to all-*trans*-β-carotene and assumes that β-zeacarotene has 25% of this value (14) and that cryptoxanthin has 57% of this value (15–17), then the all-*trans*-β-carotene equivalent of the "active" carotenes becomes 50.7 to 53.5% of the crude carotene values determined on the three samples of corn and the two samples of corn gluten meal. (It should be pointed out that some researchers (18–20) have presented data that cryptoxanthin is equal to all-*trans*-β-carotene for liver storage of vitamin A). If one further assumes that the pig is only 30% as efficient as the rat in converting all-*trans*-β-carotene to vitamin A (21), then one would expect only 15.2 to 16.0% of the crude carotenes to be converted and stored as liver vitamin A. When these percentages are multiplied by 1667 IU, which represents the theoretical vitamin A potency of 1 mg of all-*trans*-β-carotene, then 1 mg of crude carotene from corn or corn gluten meal would have a calculated potency ranging from 254 to 267 IU. These values are very close to the experimentally determined value of 261 IU/mg when corn was the only source of carotenes and the concentration of carotenes in the diet ranged from 1.05 to 5.52 mg/kg. This range of carotene concentration would be typical for fortified corn-soybean meal diets currently used for swine, and suggests that the NAS–NRC (1) relationship between 1 mg of dietary carotene and 500 IU of vitamin A exaggerates the usefulness of corn carotenes as a source of liver vitamin A for swine when these carotenes are determined by AOAC procedures (8).

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Studies of Ferritin and a New Iron-binding Protein Found in the Intestinal Mucosa of the Rat [1]

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ABSTRACT Intestinal mucosa and liver tissues from adult rats were examined for ferritin in these studies. Starch gel electrophoresis of preparations of these tissues labeled in vivo with iron-59 were stained with amido black for protein, and radioautographs were made of the gel slabs. Commercially obtained horse spleen ferritin was used as a reference standard. Results showed that intestinal mucosa contains ferritin with the same electrophoretic mobility as the alpha band of horse spleen ferritin as well as another iron-containing protein (MIBP). Liver preparations contained ferritin which corresponded to the beta band of horse spleen ferritin and did not contain MIBP. Both ferritins could be precipitated with rabbit antiferritin serum but MIBP could not.

Two concepts for iron transport through the gut wall have been advanced. According to one concept, when a surplus of iron is presented to the mucosa, the ferritin "traps" the excess and prevents its further passage into the blood supply. The "trapped" iron is lost to the body by a sloughing of the mucosal cells into the lumen and it eventually appears in the feces (1). According to the conventional exposition of this hypothesis, some protein other than ferritin must be involved in transport of the iron. A second concept (2) holds that ferritin itself may serve as the transport protein in shuttling iron from the lumen to the blood supply. If this is true, it would then seem reasonable that some other mechanism must exist to account for the "trapping" of iron.

This paper describes some studies of the physical and chemical properties of an iron-binding protein other than ferritin which is present in mucosal preparations. The electrophoresis of horse spleen ferritin, rat liver, and mucosal ferritin on starch gel is also described.

METHODS

Adult female rats of the Sprague-Dawley strain were anesthetized and an abdominal incision was made. A tracer dose of $^{59}FeCl_3$[3] was injected into the upper duodenum and two jejunal sites selected at random. A total volume of approximately 3 ml was used. The incision was then closed and the rat was allowed to recover. After a period of time (usually one hour) the rat was killed and the gut removed. Any external fat and other tissues adhering to the gut were removed, after which it was slit and washed gently in distilled water. The mucosa was then scraped off with the edge of a glass slide. When liver ferritin was isolated, the livers were perfused after removal with cold 0.15 M KCl.

The procedure used to isolate ferritin is a modification of the method of Gabrio et al. (3). The intestinal mucosa from a single rat was suspended in 30 ml of distilled water and homogenized for 45 seconds in a blender. The liver was homogenized in 50 ml of distilled water. The homogenates were heated with constant stirring in a 75° water bath for 5 minutes after which they were placed in an ice bath for 30 mintues. The resulting precipitate was removed by centrifugation at 3000 rpm for 30 minutes and discarded. The liver supernatant and mucosal supernatant solutions from a single rat were concentrated separately to a small volume (4 to 5 ml) on a flash evaporator and chromatographed on a 1.5 cm by 30 cm Sephadex [4] G-200 column using 0.9% NaCl as the

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[2] Work on this project terminated with the death of the senior author.
[3] Obtained from Abbott Laboratories, North Chicago, Ill.
[4] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

eluting medium. Ferritin, which has a molecular weight of about 500,000, is excluded by Sephadex G-200 and is thus separated from most of the contaminating protein. The mucosal and liver ferritin in the protein peak eluted just after the void volume was precipitated by saturation with $(NH_4)_2SO_4$ to 50%. The precipitates were then redissolved in water. All but traces of the excess $(NH_4)_2SO_4$ were removed by passing the solution through a 1 cm by 9 cm Sephadex G-200 column. The traces that did remain were removed by dialysis against distilled water until no ammonium ion in the dialysate could be detected (Nessler's reagent). The ferritin was stored in solution at 4° because lyophilization and freezing rendered the ferritin relatively insoluble in water.

Starch gel electrophoresis and amido black staining for protein were performed by the method of Smithies (4). The starch was prepared in 0.74 M Tris-citrate buffer, pH 9.5. A bridge buffer of 0.1 M boric acid, pH 9.5 was used (5). Radioautographs were made by placing the plastic-wrapped gel slabs against medical X-ray film in small film boxes for a period of 3 to 5 days.

Horse spleen ferritin,[5] which was used as a control in the isolation procedures for rat mucosal and liver ferritin, and rabbit antiferritin serum[6] were obtained commercially. Apoferritin was prepared by the dithionite method of Granick and Michaelis (6). Radioactive iron samples were counted in a well-type crystal scintillation counter.[7] Chemical iron assays were performed by a modification of the method of Lorber (7). Protein determinations were made by the method of Lowry et al. (8).

RESULTS AND DISCUSSION

Sephadex G-200 chromatography of both mucosal and liver preparations, which had been labeled with ^{59}Fe, showed similar patterns of radioactivity, each having a large peak which contains ferritin just after the void volume followed by a small peak (figs. 1A and 1B). Bovine hemoglobin is retained by Sephadex G-200 to about the same extent as this second peak. The identity of this second peak as rat hemoglobin has not been established, however.

Samples of Sephadex G-200 ferritin peaks from rat liver and mucosa were compared with horse spleen ferritin made radioactive by mixing it with a trace amount of ^{59}Fe, by precipitation with rabbit antiserum. While 90 to 100% of the radioactivity in Sephadex G-200 peaks containing ferritin from horse spleen or rat liver could be precipitated with antiserum, only 67 to 74% of the activity could be precipitated from the comparable peak from a mucosal preparation. These results indicated that rat intestinal mucosa contained a high-molecular-weight iron-binding material other than ferritin. When the radioactive supernatant solution from the 50% $(NH_4)_2SO_4$ precipitate of a mucosal ferritin peak was passed through a G-200 column, a peak of decreased activity appearing at the same position as the original ferritin peak was seen. This peak from various preparations was lyophilized and the protein determined. The material contained 20 to 25% protein. This material, which we call mucosal iron-binding protein (MIBP), also contained iron, hexose, and RNA in variable amounts. Rat liver and spleen ferritin preparations did not contain this substance. We believe, therefore, that this iron-binding protein is peculiar to the mucosa. Very limited studies indicate that this material is present also in hog and mouse intestinal mucosae.

Commercial horse spleen ferritin and the Sephadex G-200 peaks from rat liver, mucosa, and spleen were examined by starch gel electrophoresis (fig. 2). Commercial horse spleen ferritin produced three separate bands which were apparent after either amido black staining for protein or radioautography. The rat liver ferritin G-200 peak gave several bands with the protein stain although only one band contained radioactivity. The rat mucosal G-200 peak gave two bands with protein stain, both of which were radioactive. The major band in the mucosal preparation corresponded to the leading or alpha band of horse spleen ferritin while the other was only slightly removed from the sample origin. The latter, which stained poorly

[5] Nutritional Biochemicals Corp., Cleveland, Ohio.
[6] Pentex Laboratories, Inc., Kankakee, Ill.
[7] Nuclear-Chicago Model DS5-8 Scintillation Well Counter and a Model 132 Analyzer-Computer.

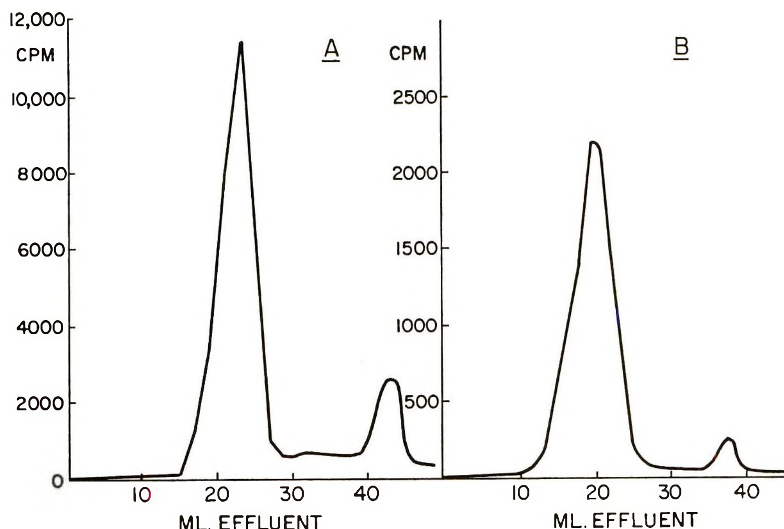

Fig. 1(A) Elution pattern of Sephadex G-200 chromatography of rat intestinal mucosa ferritin. The column (1.5 by 30 cm) was eluted with 0.9% NaCl. (B) Elution pattern of Sephadex G-200 chromatography of rat liver ferritin. The column (1.5 by 30 cm) was eluted with 0.9% NaCl.

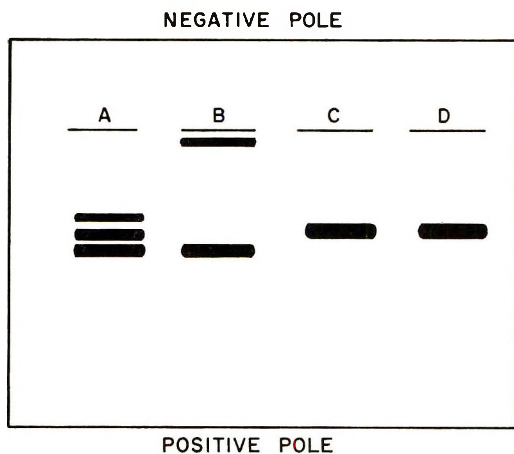

Fig. 2 Schematic of a radioautogram of starch gel electrophoresis of ferritin peaks from Sephadex G-200: (A) horse spleen; (B) rat intestinal mucosa; (C) rat liver; (D) rat spleen.

with amido black, corresponds to MIBP. Treatment of the mucosal G-200 peak with ferritin antiserum resulted in the disappearance of the prominent alpha band on electrophoresis but the MIBP band persisted. Rat spleen ferritin behaved like rat liver ferritin on electrophoresis. All ferritins were treated with dithionite to form apoferritin. Starch gel studies of these apoferritins showed all protein bands to be in the same positions as the corresponding ferritin preparations. Treatment of MIBP with dithionite resulted in the loss of virtually all of its radioactivity, but there did not seem to be an alteration in its electrophoretic movement.

Disc electrophoresis of Sephadex G-200 peaks, which had not been treated with $(NH_4)_2SO_4$, was performed by the method of Davis (9). Comparisons with horse spleen ferritin were made. Horse spleen ferritin produced one clear-cut band staining with amido black which was very close to the stacking gel. The mucosal preparation produced two bands: one corresponded to the ferritin band, and the other assumed a position about halfway down the gel column. We presume this latter band to be MIBP but it was not possible to stain it for iron or to make a radioautograph with the small amount of sample used in this technique. Many attempts were made to purify larger quantities of the two mucosal iron-binding proteins (mucosal ferritin and MIBP), using both horizontal and vertical acrylamide gel systems and preparative disc electrophoresis, but these have so far been unsuccessful.

Blanc and Isliker (10) have described a mucoprotein with characteristics similar to MIBP.

The possibility exists that the iron is only adsorbed by the MIBP, because all labeling was done via injections into the gut itself rather than parenterally. However, further work on this project was terminated by the death of the senior author and this possibility has not been explored.

Although the amount of iron bound by MIBP is significant, on the basis of these preliminary data it is not possible to assign to the MIBP a role in the storage or transport of iron.

ACKNOWLEDGMENTS

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Increases in Skeletal Calcium and Femur Cortex Thickness Produced by Undernutrition [1]

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ABSTRACT The effects of undernutrition on skeletal mass and density were studied in rats from weaning to maturity. Eight groups, each of three littermate male rats, were divided into one group that were fed ad libitum and two others that were fed the same liquid diet but were given only two-thirds of the quantity, so that growth was retarded. The undernourished animals had more total body calcium at any given body weight than the controls. The long bones were of the same volume as the controls, but had thicker cortices and appeared to sustain a greater load in compression. It is concluded that maximal growth rate may be incompatible with optimal skeletal characteristics.

Optimal nutrition is difficult to define, but there is a tendency to equate it with maximal growth rate, not only in domestic animals (used for their meat) but also in children. The effects of inadequate calcium intake in animals are well known (1, 2). Furthermore, Bell et al. (3) showed that in rats, increasing calcium intake resulted in enhanced bone density and bone strength up to a threshold level above which no further improvement in these parameters occurred despite increasing calcium in the diet. The purpose of this report is to show the effect on the skeleton of reducing the quantity of a nutritionally adequate diet to the extent that growth rate was impaired while maintaining the quality of the diet including calcium intake. In short, we are reporting the skeletal changes in undernourished animals.

MATERIALS AND METHODS

Male rats of the Charles River C. D. strain were purchased in eight groups of three weanling littermates each. They were housed in individual wire-bottom cages and were given liquid diets in graduated drinking tubes as the only source of food and water, as described elsewhere (4). All rats received the same adequate diet containing 16% of total calories as amino acids, 43% as fat and 41% as sucrose. The calcium content was 1.3 mg/kcal, well above the threshold value of about 1 mg/kcal below which, according to Bell et al. (3), dietary calcium changes influence calcium retention by the body (including calcium content, and bending and twisting strength of bone). In each group of three animals, one was given the diet ad libitum whereas the two others received two-thirds of that amount. During the first 8 weeks, four animals were killed every other week: two of the rats given the diet ad libitum and two corresponding "limited" littermates. After 8 weeks, the eight remaining "limited" rats were continued on the limited regimen and every other week, two of these rats were killed. There was no mortality during the experiment. The body weight of the rats was determined at least twice a week. The animals were killed by decapitation and eviscerated. The carcasses were immediately frozen and stored at −18° until analysis.

The carcasses were disarticulated at the hips and shoulders. One femur and one humerus were divested of soft tissue, sus-

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pended in distilled water at 4° by a fine copper wire and degassed for 0.5 hour in a vacuum chamber. The bones were then weighed while suspended in the distilled water, mopped dry and reweighed in air. The volume and specific gravity of the femur and humerus were calculated by Archimedes' principle. The limb bones, as well as the trunk were ashed separately in a muffle furnace at 600° for 24 to 48 hours. Each sample was then dissolved in 6 N HCl and its calcium content determined by a semiautomatic EDTA method (5).

Breaking-force measurements were performed on the whole right humerus and on that shaft section of the right femur which lies between the ridge of the third trochanter and the condyles, by methods previously described (6).

It has been shown in these laboratories that the weight, volume, density, breaking force and calcium content of the long bones as well as the total calcium of the rat are all linear functions of body weight (6).

The regressions of these variables on body weight were calculated by the method of least squares and they were compared one with another by analysis of covariance (7).

RESULTS

The growth curves of the restricted and control animals are shown in figure 1, and the regression analyses are summarized in table 1. While, at any given age, total body calcium was greater in the larger control animals compared to the restricted animals, when total body calcium was considered as a function of body weight the restricted animals were found to have significantly greater total calcium than the controls (fig. 2). Similar findings applied to the various component parts of the skeleton —the axial skeleton, femur and humerus calcium as functions of body weight were all greater in the restricted animals. Femur volume was found to be identical in the restricted and the control animals at any given body weight, but humerus and femur calcium content were significantly greater in the restricted animals, when considered as a function of bone volume or of body weight. Figure 3 illustrates this point when femur specific gravity is considered as a function of weight. However, there was no difference between the restricted and control animals when calcium was considered as a percentage of bone weight. The reason for these findings is apparent in figure 4 which shows that the femoral shaft —which is, in effect, a hollow cylinder— has a thicker cortex in the restricted ani-

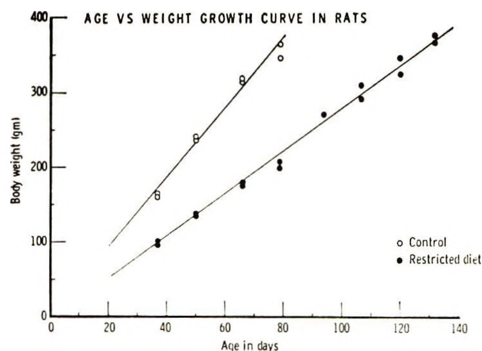

Fig. 1 Growth curves for control and restricted animals.

TABLE 1

Statistical summary

Regression Y versus X	F ratio (adjusted means)	Significance
Femur calcium versus body weight	37.93	$P < 0.001$
Femur calcium versus femur weight	0.24	NS
Humerus calcium versus body weight	48.37	$P < 0.001$
Humerus calcium versus humerus weight	0.45	NS
Axial skeletal calcium versus body weight	26.08	$P < 0.001$
Total calcium versus body weight	35.77	$P < 0.001$
Femur breaking force versus body weight	2.4	NS
Humerus breaking force versus body weight	3.42	NS
Femur specific gravity versus body weight	28.8	$P < 0.001$
Femur thickness versus body weight	9.64	$P < 0.01$

[1] Regressions for each pair of variables listed were calculated for controls and restricted animals. The adjusted means were then compared; the *F* ratios and *P* values are listed.

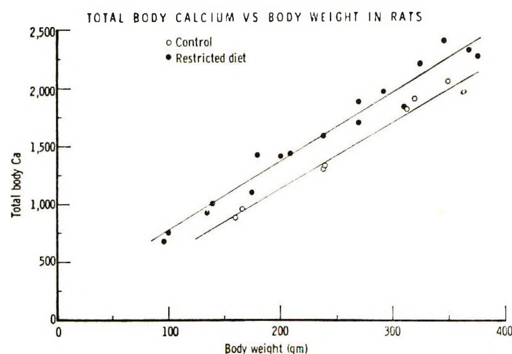

Fig. 2 Total body calcium (mg) as a function of body weight. There is a highly significant difference in the adjusted means.

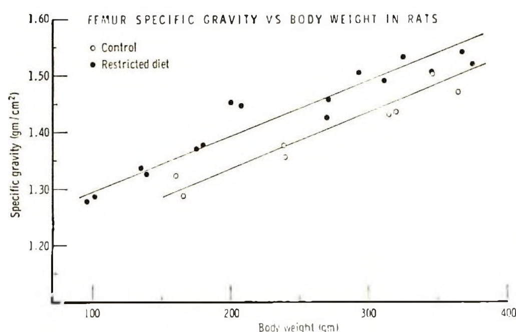

Fig. 3 Femur specific gravity as a function of body weight. There is a highly significant difference in adjusted means between the control and restricted animals.

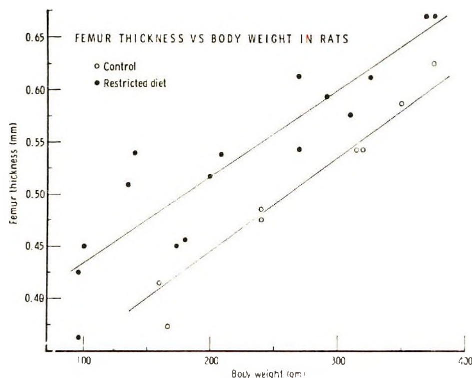

Fig. 4 Restricted animals have thicker femoral cortices at any given body weight compared to controls. Each point represents the average of three measurements.

mals compared with the controls. Thus, restricted rats at any age are smaller than controls and have less total calcium but when considered on a body-weight basis they have more calcium. The long bones are of similar volume and are similarly mineralized but the cortices are thicker in the restricted animals, hence, the calcium content of these long bones is greater.

Strength testing showed that the femora and humeri of animals on a restricted diet could sustain a greater load in compression than the control bones. However, these differences did not attain statistical significance, probably because of the imprecision of the measurements relative to gravimetric and chemical determinations.

DISCUSSION

The extensive studies of McCay and coworkers (8) have shown that reduction in calories, primarily carbohydrate and fat increased longevity in rats. Fairbanks and Mitchell (9) showed that the calcium content of the rat carcass decreased with increasing growth rate. In the study reported here we have restricted all constituents of the diet. We have found that the calcium content of the axial skeleton and the appendicular skeleton is increased in these undernourished animals. This increase in mineral content of the long bones is due, not to a change in mineralization of the organic matrix, but rather to the presence of thicker cortical bone without change in total external bone volume. The shafts of long bones of growing animals enlarge by apposition of bone on the subperiosteal surface coincident with resorption from the endosteal surface. The two processes seem to be linked, so that a bone retains its proper thickness and appropriate shape. Increase in cortex thickness without change in external volume indicates inhibition of endosteal bone resorption in the restricted animals. We did not measure the percentage of calcium of the axial skeleton but we see no reason to believe that it would be different from the appendicular skeleton, because the total calcium content of the axial skeleton and of the skull of the undernourished animals was considerably greater at any given body weight than that of the control animals. Therefore, decreased bone resorption is, presumably, not confined to the endosteum of long bones. These quantitative changes would be expected to lead to

decreased fragility of bone and this trend was observed. Maximal growth rate may be incompatible with longevity, as has been shown in the past, and also with maximal strength and density of the skeleton, as reported in this study.

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Amino Acid Levels and Enzyme Activity in Tissues of Rats Force-fed Diets Differing in Methionine Content [1,2]

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ABSTRACT Rats weighing about 200 g were force-fed diets containing amino acid mixtures proportioned as in casein (21% of diet) but with varying amounts of methionine for a 3-day period. One group received a complete amino acid mixture (CAA). Other groups were given CAA diet devoid of methionine and cystine (−S) or CAA diet supplemented with 2% (+2% Met) or 4% (+4% Met) methionine. Plasma, liver and gastrocnemius muscle of the three groups of animals were analyzed for amino acids, and various hepatic amino acid-degrading enzymes were measured. With the −S diet as compared to the CAA diet, the methionine content of the tissues was similar but the cystine level in plasma was greatly decreased, and some other amino acids were increased, as for example, threonine and arginine. With the +4% Met diet as compared to the CAA diet, methionine was elevated in all tissues but a number of other amino acids were decreased including ornithine, arginine, glycine and glutamic acid. The activity of the amino acid-degrading enzymes in liver tissue tended to be lower with −S diets than with CAA diets and higher with +4% Met diets. For the −S diets a low threonine dehydratase activity was associated with high tissue concentrations of threonine and serine. The converse was found with +4% Met diet: high enzyme activity and low concentrations of the same amino acids.

A number of recent experiments have described alterations in amino acid concentrations in blood and tissues resulting from a deficiency of one or more amino acids in the diet. Some have been studies of single amino acid deficiencies in which measurements have been made of pathological changes, amino acid concentrations and, in a few instances, enzyme activities in various tissues (1–6).

This study represents a search for related alterations in levels of tissue amino acids and in the activity of hepatic enzymes catabolizing amino acids of rats force-fed diets without the sulfur-containing amino acids. Since there are many reports of methionine toxicity (7–11), some groups of animals were fed excess amounts of methionine and the same experimental parameters were measured. The hepatic enzymes participating in amino acid catabolism that were studied included methionine-activating enzyme, cystathionase, tryptophan pyrrolase, tyrosine transaminase, threonine dehydratase and glutamic-oxaloacetic transaminase.

EXPERIMENTAL

As in a previous study with leucine, isoleucine, valine and threonine deficiencies (4), all animals were force-fed the amino acid-containing diets for 3-day periods. A food supply adequate in calories and constant in all nutrients except the amino acid under investigation was thus maintained.

Male rats (210 to 230 g) of the Sprague-Dawley strain were allotted at random to the various diets. The control animals received the complete amino acid (CAA) diet as previously described (4). This diet contained 21.5% of an amino acid mixture proportioned as in casein including 0.56% L-methionine and 0.27% L-cystine. The experimental animals received diets similar to CAA but devoid of methionine and cystine (−S). Some animals received the CAA diet with an additional 2% or 4%

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[2] Part of this study is taken from a thesis presented by A. Sanchez in partial fulfillment of requirements for the Doctor of Public Health degree and is abstracted in Federation Proc., 27: 613, 1968.

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of L-methionine as a supplement (+2% Met and +4% Met).

The experimental procedures of tube feeding and handling the animals were as previously described (4) with the animals receiving 20 g of a diet (5 g/feeding) each day for a 3-day period. The animals were killed 8 hours after the final meal, a time period found to represent a postabsorptive steady state concentration of amino acids in plasma under these experimental conditions.

The blood was removed by heart puncture using heparinized syringes. After centrifugation, the plasma was deproteinized with a fivefold amount of 3% sulfosalicylic acid. The liver and gastrocnemius muscle were quickly excised and 1 g of liver was weighed, minced and deproteinized immediately in a Potter-Elvehjem homogenizer with a 10-fold amount of the sulfosalicylic acid solution. This portion was used for amino acid analyses. The remainder of the liver was frozen on dry ice and all samples were stored at −20° until used for enzyme analyses. The muscle was ground in a Virtis homogenizer with a 20-fold amount of sulfosalicylic acid. All samples were centrifuged and the supernatants stored at −20° until analyzed.

Amino acid analyses were performed by ion-exchange chromatography (12) on deproteinized supernatants of plasma, liver, and muscle using an amino acid analyzer.[4] Asparagine and glutamine were not separated chromatographically and were tabulated as a mixture. The experimental procedures thus far used are the same as those reported previously from this laboratory (4).

The hepatic enzymes measured and the methods used were as follows: methionine-activating enzyme, method of Cantoni and Durell (13); cystathionase, method of Greenberg (14); threonine (serine) dehydratase, method of Ichikawa et al. (15); tryptophan pyrrolase, assayed according to Knox (16); tyrosine transaminase, method of Rosen et al. (17); and glutamic-oxaloacetic transaminase, the method of La Due et al. (18) adapted for liver. Statistical analyses were according to the Scheffé multiple comparisons method (19).

RESULTS

The results of 3-day studies with groups of animals fed CAA, −S, or +4% Met diets represent duplicate experiments in all instances.

In table 1, data on body, muscle and liver weights and the nitrogen content of liver for the various experimental groups are given. Animals fed either the sulfur-devoid or the +4% Met diets lost weight while rats on the CAA diet and the +2% Met diet gained. There was a direct relationship between liver weight and dietary methionine content whereas the muscle weight remained relatively constant. The total liver nitrogen was proportional to the dietary methionine levels. The values for percentage of nitrogen and the total nitrogen of the liver were significantly less in rats fed the −S diet than values for the other groups of animals.

[4] Beckman Instruments, Inc., Spinco Division, Palo Alto, California.

TABLE 1

Body, muscle, and liver weights and nitrogen content of rats force-fed diets varying in methionine content [1]

Diet [2]	No. of samples [3]	Body weight change	Muscle weight [4]	Liver		
				Weight	N content	Total N
		g	g	g	%	mg
−S	5	− 7.0 ** [5]	0.42	7.0	3.2 **	223 **
CAA	6	+10.1	0.44	7.8	3.6	281
+2% Met	5	+ 5.0	0.42	8.3	3.5	290
+4% Met	6	−10.2 **	0.40	9.2 **	3.4	312

[1] All values are sample means.
[2] The test diets are described in the text and are as follows: Complete amino acid diet (CAA), CAA less sulfur amino acids (−S), CAA supplemented with 2 g (+2% Met) or 4 g (+4% Met) of L-methionine per 100 g diet.
[3] Each sample represents a pool from two animals.
[4] Right gastrocnemius muscle.
[5] Significant difference from the CAA diet values: ** $P < 0.01$ by Scheffé multiple comparisons method (19).

The amino acid levels of plasma, liver and muscle as measured 8 hours postprandial for animals receiving the various diet treatments are shown in table 2. Only those amino acids were tabulated for which values in one or more of the experimental groups differed significantly from those in the group given the CAA diet.

In animals fed diets lacking the sulfur-containing amino acids, methionine concentrations in plasma and tissues did not differ significantly from those of control animals but cystine in plasma was greatly reduced. Even though methionine levels were not altered, other amino acid concentrations were changed by feeding this diet. Ornithine and arginine tended to be increased in plasma and tissues as compared to values for control animals. Threonine and serine also showed significant elevations in some tissues under the same circumstances.

With diets containing excess methionine, there were alterations in the concentrations of a number of amino acids, particularly in muscle tissue, as compared with those of animals given the CAA diet. With the +4% Met diet, methionine was significantly elevated in all tissues. Histidine, alanine and α-aminobutyric acid (α-ABA) were likewise increased in plasma and liver, but other amino acids showing changes were reduced in concentration in one or more of the tissues measured. Glycine was decreased in all tissues. Serine and threonine were not significantly decreased due to the great variability between samples; there was an indirect relationship, however, between the concentrations of these amino acids and the level of dietary methionine. Other amino acids that were significantly lower in concentration after feeding the +4% Met diet included glutamic acid, aspartic acid, glutamine, citrulline, ornithine, arginine and lysine.

The data on the activity of the various enzymes that were measured are given in table 3. Although the methionine-activating enzyme and cystathionase showed no significant differences among the dietary groups, the activities of both enzymes tended to increase with increasing amounts of dietary methionine. The same pattern was manifest for threonine dehydratase. For this enzyme, there was a significant increase in activity with the +4% Met diet as compared to the CAA diet, whereas with the sulfur-devoid diet, although there was a substantial reduction in enzyme activity, significance could not be demonstrated because of the wide variation in sample values. Glutamic-oxaloacetic transaminase was also significantly decreased with the sulfur-devoid diet. Both tryptophan pyrrolase and tyrosine transaminase were significantly elevated with the +4% Met diet as compared to the CAA diet.

DISCUSSION

These experiments with diets containing either deficient amounts or excesses of methionine, wherein force-feeding was employed to maintain a constant food intake, show that animals lose weight and have alterations in liver weight and nitrogen content as compared to rats fed a complete amino acid mixture. Comparable results have also been obtained in animals allowed similar diets ad libitum (3, 7, 8). The toxic manifestations of these diets can now be extended to include alterations in specific amino acid concentrations of tissues and in hepatic amino acid-degrading enzymes.

Perhaps the most unexpected finding was that, despite a 3-day feeding period of a diet completely lacking in both methionine and cystine, the methionine levels in the tissues remained similar to those of animals fed the CAA diet. The plasma cystine, however, was much lower in animals fed the −S diet. These results suggest that a reduced cystine level might be a better indicator of a methionine-deficient diet than the methionine concentration itself, at least within dietary conditions that include adequate caloric intakes. It might be presumed that the stable methionine concentration resulted from a reduction in the catabolic rate of this amino acid. In vitro measurements of the methionine-activating enzyme, however, indicated no change in activity. Likewise, cystathionase activity was not greatly affected. Despite the stability of the methionine level in tissues, the levels of other amino acids were altered when −S diets were fed. Except for cystine, in general these alterations tended to be in the direction of elevated amino acid

TABLE 2

Amino acid levels in plasma, liver and muscle of rats force-fed the indicated diets [1]

Amino acid	Plasma				Liver				Muscle			
	– S [2] Diet (5) [3]	CAA Diet (6)	+ 2% Met Diet (4)	+ 4% Met Diet (5)	– S Diet (4)	CAA Diet (6)	+ 2% Met Diet (5)	+ 4% Met Diet (6)	– S Diet (5)	CAA Diet (6)	+ 2% Met Diet (5)	+ 4% Met Diet (6)
	µmoles/100 ml				*µmoles/100 g fresh tissue*				*µmoles/100 g fresh tissue*			
Essential [4]												
Met	3.4	3.1	7.6	191.0 ** [5]	6.6	8.5	11.3	171.8 **	11.7	7.1	9.7	239.2 **
Half-Cys	1.7 **	6.9	8.2	11.1								
Thr	46.2	36.9	35.9	35.8	114.0 **	35.5	37.4	31.5	156.0	118.8	97.3	75.6
Lys	61.6	57.8	81.2	56.8	92.4	76.9	97.0	87.3	207.1	268.5	304.2	70.4 **
His	5.2	4.1	6.8	8.0 **	45.1	45.5	61.2	78.5 *	25.7	27.5	20.0	21.7
Nonessential												
Ser	46.2	35.6	29.9	33.0	327.4 **	94.1	61.0	16.4	162.6	184.2	148.2	69.2
Gly	25.1	30.8	22.4 **	20.0 **	243.3	232.4	189.9	77.4 **	284.9	421.1	215.7 **	168.9 **
Asp					102.9 *	204.3	157.6	107.7 *	23.9	33.3	28.1	15.7 *
Glu-NH_2	15.9	5.9	2.6	17.7	559.3	622.8	590.0	411.4 *	295.5	337.9	232.3 *	124.9 **
Glu	12.5	12.3	13.6	14.7	178.3	255.6	244.0	215.7	204.1	216.2	188.0	114.2 **
Cit	8.8	7.9	6.5	6.8					20.3	23.5	15.2	8.0 **
Orn	5.3 **	3.6	4.0	3.9	34.1 **	19.8	18.3	22.2	7.7	10.3	4.7 *	4.5 *
Arg	17.0 **	10.8	15.4 **	12.0	1.1	1.0	1.5	1.2	65.3 *	47.7	43.0	16.5 **
Ala	43.1	53.5	45.5	78.2 **	331.9	356.9	389.6	549.6 *	331.7	347.3	265.9	291.5
α-ABA [6]	3.9	3.2	9.2	12.4 **	19.9	11.7	20.8	37.5 **	6.1	6.6	13.8	13.3

[1] All values are sample means.

[2] The test diets are described in table 1.

[3] Numbers in parentheses indicate number of samples, each representing a pool from 2 animals.

[4] Includes cystine.

[5] Significant differences from the CAA diet values: * $P < 0.05$; ** $P < 0.01$, by Scheffé multiple comparisons method (19).

[6] α-Amino butyric acid.

TABLE 3

Hepatic enzyme activities of rats force-fed diets varying in methionine content

Diet [2]	No. of samples [3]	Avg enzyme activity (units/gram fresh liver) [1]					
		Thr (Ser) dehydratase	Met activating enzyme	Cystathionase	Trp pyrrolase	Tyr transaminase	Glu oxaloacetic transaminase
−S	5	114	46	107	2.3	65	173 ** [4]
CAA	6	488	47	133	2.2	32	241
+2% Met	5	692	49	134	2.9	40	203
+4% Met	6	1082 **	52	139	3.4 **	95 **	249

[1] All values are sample means. One unit for each respective enzyme is as follows: 1 μmole of α-ketobutyric acid formed/hr (Thr dehydratase); 1 μmole S-adenosyl methionine formed/30 min (Met activating enzyme); 1 μmole α-ketobutyric acid formed/hr (cystathionase); 1 μmole kynurenine formed/hr (Trp pyrrolase); 1 μmole *p*-hydroxyphenylpyruvic acid formed/hr (Tyr transaminase); international unit calculated from ΔA/min (Glu oxaloacetic transaminase).

[2] The diets are described in table 1.

[3] Each sample represents a pool of 2 animals.

[4] Significant differences from the CAA diet: ** $P < 0.01$ by Scheffé multiple comparisons method (19).

levels, as for example with ornithine and arginine.

When dietary excesses of methionine were administered, particularly with the +4% Met diet, methionine was elevated in the tissues, and other amino acid levels were also affected. With few exceptions, these alterations tended to be in the direction of decreased levels. Ornithine and arginine, for example, were reduced in muscle of methionine-toxic rats as compared with control animals. With the −S diet the effect was found to be opposite in muscle, and the levels of ornithine and arginine were elevated in the other tissues as well.

A general pattern also seems to apply to the activity of the amino acid-catabolizing enzymes in relation to the dietary methionine content. With a methionine-devoid diet the activity of some of the enzymes (such as threonine dehydratase and glutamic-oxaloacetic transaminase) was low, whereas with an excess methionine diet the activity of all enzymes studied, except glutamic-oxaloacetic transaminase, was enhanced. It should be noted that this increased hepatic enzyme activity was associated with a significant increase in total liver nitrogen (table 1).

In view of the fact that the activities of some of the amino acid-degrading enzymes are directly related to the dietary methionine content, there is the possibility that methionine acts as an inducer for these enzymes. However, because in the animals receiving the −S diets the methionine levels in tissues approximate those of control animals, it is also possible that some metabolic product of methionine, such as cystine which is decreased in plasma, is the primary inducer. Methionine has been shown to increase blood corticosterone levels (20); thus, this amino acid or a metabolite may act through the adrenal cortex to promote the induction of enzymes (21) or reduce their rate of degradation (22).

In this study with the varying dietary methionine content, there was an association of low amino acid levels in tissues with high enzyme activity, suggesting that the increased enzyme activity is responsible for the decreased amino acid concentration. The converse was also found. For serine dehydratase there was a highly significant negative correlation ($P < 0.01$) with the tissue levels of serine, i.e., as the enzyme activity increased the substrate level in the tissue decreased. The glycine level was also reduced with excess methionine diets, and because glycine can readily be converted to serine, it is possible that the low level of serine limits the availability of glycine either by decreasing synthesis or by increasing conversion to serine.

Other investigators (9, 11) have observed that the toxic effects of excess dietary methionine on limiting the growth rates of rats can be overcome by dietary supplementation with glycine or serine. The present studies suggest a possible explanation for these observations: high dietary levels of methionine produce a tissue deficiency of glycine and serine secondary to alterations in the activity of enzymes necessary for their metabolism, such as threonine

dehydratase. It has been observed[5] that, 8 hours after feeding a single meal of +4% Met diet, glycine and serine concentrations in rat liver are reduced and that the serine dehydratase shows some elevation, though not significant as compared to values in rats fed CAA diet.

These studies of associated changes in tissue amino acid levels and hepatic enzyme activities with a varying methionine dietary content indicate that suboptimal or excessive intakes of one amino acid may alter the tissue availability of another amino acid indirectly through an effect on enzyme activity, and that it is possible in this fashion to produce a tissue deficiency of a nonessential amino acid. A study of dietary deficiencies and excesses of various single essential amino acids may uncover other such relationships. It has been observed (4) that dietary leucine deficiency and excess result in changes in tissue concentrations of a number of amino acids, and it is possible that this amino acid also affects, directly or indirectly, the activity of enzymes relating to amino acid metabolism.

In the present study with varying dietary methionine content, there are alterations in amino acid levels other than those already discussed which might also be related to changes in the activity of some associated enzyme. Relatively high levels of ornithine and arginine in tissues of animals on low methionine diets, and the reverse condition in muscle of animals with excess methionine intake, may result from alterations in the urea cycle enzymes, because these are known to be influenced by diet and more specifically by dietary protein content (23). Likewise, the elevated histidine levels in plasma and liver of animals receiving +4% Met diets may result from some alteration in the metabolism of single carbon atoms since histidine is degraded via a formylated derivative (24).

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A Study of the Relative Potency of L- and D-Thyroxine in Preventing Kidney Calcification Associated with Magnesium Deficiency [1]

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ABSTRACT A series of three studies was conducted, using young male albino rats, to investigate the relative effectiveness of D- and L-thyroxine (T_4) in prevention of kidney calcification induced by magnesium deficiency. In experiment 1, 10 ppm of either T_4 isomer in the diet prevented kidney calcification although D-T_4 inhibited weight gains less than did L-T_4. Experiment 2 showed that D-T_4 either injected or fed on alternate days was less effective than L-T_4 in anticalcifying action. In experiment 3 it was shown that 10 μg D-T_4 injected daily in 5 divided doses was as effective as 10 μg L-T_4 injected once daily. The data indicate approximate equivalance of action of the isomers in anticalcifying effect only if D-T_4 is administered frequently enough to offset its more rapid metabolism in the tissue.

In a previous study (1) we reported that kidneys of magnesium-deficient rats were protected against calcification by intraperitoneal injections of L-thyroxine (L-T_4) at 48-hour intervals but that D-thyroxine (D-T_4) was not similarly effective. Differences in relative potency of these isomers have been reported in a variety of species and by various criteria. D-T_4 has been found to be one-half to one-tenth as active as L-T_4 for calorigenesis in man and rats (2), one-fifth as active as L-T_4 in increasing liver mitochondrial α-glycero-phosphate dehydrogenase at 24 hours, but equal to L-T_4 at 12 hours, after administration (3), and equal to L-T_4 in alleviating myxedema (4) and in lowering blood cholesterol (5). In the latter instance D-T_4 produced this effect without increasing metabolic rate and appearance of cardiac complications associated wtih L-T_4 treatment.

These differences in physiologic activity have been attributed to: a) a more rapid rate of inactivation and metabolism with the resultant shorter half-life of D-T_4 in blood than L-T_4 (6, 7); b) a decreased binding affinity of D-T_4 for serum thyroxine-binding protein compared with that of L-T_4 (8); and c) the mode of administration (Garvin and Galysh [3]).

The investigations to be described were undertaken with the aim of clarifying the apparent difference in activity of these isomers on the kidney calcification induced by magnesium deficiency.

METHODS

Young male albino rats of the Sprague-Dawley strain were used in these experiments. The basal diet composition is shown in table 1. Changes in $MgCO_3$ levels were made in the mineral premix to obtain the required concentration of magnesium in the final diet. Demineralized water was supplied in all instances. Daily food intake and weekly body weights were recorded.

At the termination of each experiment the animals were anesthetized with ether and blood samples were obtained from the abdominal aorta. Kidneys were also obtained at this time. Mineral analyses were made by atomic absorption procedures using standard methods. The data were treated statistically by analysis of variance and Tukey's test when applicable. Significant statistical differences cited in the text are at the 5% level or less.

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TABLE 1

Composition of the basal diet

	%
Casein [1]	15.0
DL-methionine	0.5
Corn oil	8.0
Cod liver oil [2]	0.5
Cellulose [3]	3.0
Vitamin mix [4]	5.0
Salt mix [5]	2.66
Glucose [6]	65.34

[1] "Vitamin-free" test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] Parke-Davis Standardized Cod Liver Oil containing 2000 USP units of vitamin A per gram and 250 USP units of vitamin D per gram.
[3] Solka Floc, Brown Company, Boston.
[4] Contained: (in mg/kg of mixture) thiamine·HCl, 200; riboflavin, 120; pyridoxine·HCl, 80; Ca pantothenate, 320; biotin, 4; nicotinic acid, 500; folic acid, 10; vitamin B_{12}-mannitol, 400 B_{12} present at 0.1%); (in g/kg mixture) choline·HCl, 30; menadione, 7; and glucose to make 1000 g.
[5] Contained: (in percent of mixture) NaCl, 14.0; K_2CO_3, 18.90; $FeSO_4 \cdot 7H_2O$, 1.50; $MnSO_4H_2O$, 0.454; $CuSO_4 \cdot 5H_2O$, 0.227; KI, 0.014; $ZnCO_3$, 0.074; $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 1.03; $CaHPO_4$, 63.801. Mg content in basal diet = 100 ppm.
[6] Cerelose, Corn Products Refining Company, New York.

Experiment 1 was designed to study the activities of D- and L-T_4 fed at a constant level of 10 ppm to magnesium-deficient rats. D- and L-thyroxine were added to the basal diet to give a final concentration of 10 ppm. Thirty weanling rats (41 to 55 g) were fed the appropriate diets for 3 weeks.

Experiment 2 was planned to investigate the relative effectiveness of D-T_4 in preventing kidney calcification when administered in the feed or when injected into magnesium-deficient rats. Eighty weanling rats (33 to 43 g) were fed 8 g each of a magnesium-deficient (64 ppm) diet for 14 days. Intraperitoneal injections of L- or of D-T_4 were given once daily on alternate days at levels indicated in table 3. Diets containing D-T_4 were fed to animals of groups 9 and 10 on the days when the rest of the animals were injected with the hormone. The animals were killed at the end of 2 weeks.

A preliminary trial following experiments 1 and 2 indicated that D-T_4 prevented kidney calcification when it was injected at 3-hour intervals or when it was fed constantly at a level of 5 ppm. Experiment 3 was designed to quantitate the minimal level and frequency of intraperitoneal administration of D-T_4 which would inhibit kidney Ca deposition in animals fed a magnesium-deficient diet. Forty animals (64 to 78 g) were fed 15 g daily of a magnesium-adequate diet (625 ppm) for a week and then given the same quantity of a magnesium-deficient (4.9 ppm) diet for the next 5 days. The injection routine is indicated in table 4.

RESULTS

Experiment 1. The data obtained in this initial study are shown in table 2. Both magnesium deficiency and T_4 treatment reduced average daily gains, the effect of T_4 being additive to that of magnesium deficiency. The calorigenic effect of D-T_4, as evidenced by the weight gain data, was less than that of L-T_4. Both D- and L-T_4 increased serum magnesium, there being no difference in this response between forms of T_4 at either magnesium level, although the serum magnesium was much reduced in the magnesium-deficient animals. All additions to the basal magnesium-deficient ration prevented calcium accumulation in the kidney.

TABLE 2

Effect of dietary L- and D-thyroxine on weight gain, food intake and tissue mineral concentration in a 3-week period

Treatment	Mean food intake	Mean weight gain	Serum Mg	Serum Ca	Kidney Ca
	g/day	*g/day*	*mg/100 ml*	*mg/100 ml*	*mg/g dry tissue*
−Mg (100 ppm)	11.6 ± 0.23 [1]	4.0 ± 0.09	0.59 ± 0.03	11.4 ± 0.29	5.94 ± 0.57
−Mg + L-T_4 [2]	11.9 ± 0.21	2.4 ± 0.06	0.78 ± 0.08	10.3 ± 0.21	0.35 ± 0.01
−Mg + D-T_4 [2]	10.9 ± 0.29	2.9 ± 0.17	0.72 ± 0.03	11.0 ± 0.26	0.43 ± 0.03
+Mg (500 ppm)	13.6 ± 0.70	5.2 ± 0.30	2.07 ± 0.04	10.9 ± 0.18	0.39 ± 0.03
+Mg + L-T_4	14.1 ± 0.41	3.4 ± 0.11	2.25 ± 0.07	10.1 ± 0.13	0.35 ± 0.01
+Mg + D-T_4	13.3 ± 0.34	4.0 ± 0.22	2.30 ± 0.05	10.6 ± 0.17	0.36 ± 0.02

[1] Mean ± SEM (5 rats per treatment).
[2] L-T_4, D-T_4: Sodium salt of thyroxine, Sigma Chemical Company. Each salt at 10 ppm of the diet.

Experiment 2. The data of this experiment are shown in table 3. Injected L-T_4 lowered weight gain below that of the basal group. Neither of the levels of D-T_4 which were fed on alternate days affected weight gain, but the higher levels of injected D-T_4 did decrease weight gain significantly. Serum magnesium was increased significantly above that of the nontreated animals by L-T_4 treatment. Injection of D-T_4 at levels of 40 μg or more generally increased serum magnesium as compared to that of the nontreated deficient rats. Parenteral administration at lower levels or dietary intake of D-T_4 did not increase serum magnesium significantly. L-Thyroxine treatment decreased serum calcium significantly from that of nontreated, magnesium-deficient animals whereas the decrease caused by any of the D-T_4 treatments was not significant. Serum phosphorus was not affected by any treatment. Kidney calcium was lowered by D-T_4 injection at all levels except the 20-μg level. The calcification was not completely eliminated, however, compared with the L-T_4-treated rats. Twenty micrograms of D-T_4, whether fed on alternate days or injected, were not effective in preventing calcification whereas the 80-μg dose lowered kidney calcium to the same extent independent of its mode of administration.

Experiment 3. As shown in table 4, D-T_4 depressed weight gains when injected in amounts greater than 10 μg daily, and the effect of L-T_4 given at the same dose level was significantly greater than that of D-T_4. Serum calcium and magnesium were not influenced by treatment. All treatments, except 10 μg D-T_4 given at 8-hour intervals, increased serum phosphorus above that found in the basal group. The injection of L-T_4 was significantly greater in its effect on serum phosphorus than were lower levels of injected D-T_4. All treatments were effective in preventing accumulation of calcium in the kidneys although five of the ten animals in groups given 10 and 30 μg D-T_4 at 8-hour intervals showed definite evidence of kidney calcification (1.15 to 2.90 mg/g).

DISCUSSION

On the basis of these results it is clear that L-T_4 produced a striking decrease in rate of growth independent of the method of administration and level of the hormone given. In contrast, D-T_4 caused a reduction in growth rate which depended on the manner, frequency and amounts in which it was administered. Daily dietary intake of D-T_4 at levels as low as 10 ppm produced a reduction in weight gain nearly comparable to that obtained with L-T_4, although feeding it on alternate days had no effect on weight gain. Intraperitoneal administration of D-T_4 on alternate days lowered body weights though not as severely as did a similar schedule of L-T_4 injection. These observations are consistent with pre-

TABLE 3

Effect of graded doses of D-*thyroxine as compared with* L-*thyroxine on serum and kidney mineral levels of magnesium-deficient rats in a 2-week period*

Treatment	Mean weight gain	Serum			Kidney Ca
		Mg	Ca	P	
	g/day	*mg/100 ml*			*mg/g dry tissue*
1 −Mg (64 ppm)	3.5 ± 0.10 [1]	0.70 ± 0.05	10.07 ± 0.40	9.4 ± 0.23	6.56 ± 1.42
2 −Mg + L-T_4 (20 μg ip)	2.4 ± 0.11	0.84 ± 0.04	8.90 ± 0.22	10.5 ± 0.22	0.60 ± 0.08
3 −Mg + D-T_4 (20 μg ip)	3.2 ± 0.03	0.77 ± 0.07	9.67 ± 0.23	9.8 ± 0.16	4.61 ± 1.07
4 −Mg + D-T_4 (30 μg ip)	3.1 ± 0.11	0.83 ± 0.06	9.47 ± 0.11	10.2 ± 0.34	2.43 ± 0.50
5 −Mg + D-T_4 (40 μg lp)	3.0 ± 0.04	0.95 ± 0.10	9.64 ± 0.12	10.9 ± 0.37	1.69 ± 1.06
6 −Mg + D-T_4 (50 μg ip)	2.9 ± 0.08	0.91 ± 0.09	9.57 ± 0.37	10.3 ± 0.27	2.27 ± 0.86
7 −Mg + D-T_4 (60 μg ip)	2.9 ± 0.09	0.83 ± 0.07	9.32 ± 0.19	9.8 ± 0.22	2.06 ± 0.90
8 −Mg + D-T_4 (80 μg ip)	2.8 ± 0.04	0.94 ± 0.07	9.25 ± 0.12	10.5 ± 0.27	1.33 ± 0.43
9 [2] −Mg + D-T_4 (20 μg)	3.5 ± 0.08	0.74 ± 0.04	9.91 ± 0.11	10.3 ± 0.31	4.80 ± 1.80
10 [3] −Mg + D-T_4 (80 μg)	3.3 ± 0.08	0.68 ± 0.03	9.31 ± 0.20	10.4 ± 0.29	1.34 ± 0.58

[1] Mean ± SEM (8 animals per treatment). Intraperitoneal (ip) injections of D- and L-T_4 given on alternate days starting with the first day.
[2] D-T_4 added to the diet so that there was 20 μg/8 g diet.
[3] D-T_4 added to the diet so that there was 80 μg/8 g diet.

TABLE 4

Effect of multiple doses of D-thyroxine on serum and kidney mineral levels of magnesium-deficient rats

Treatment	Mean weight gain	Serum Mg	Serum Ca	Serum P	Kidney Ca
	g/day	*mg/100 ml*			*mg/g dry tissue*
1 —Mg + 10 μg L-T_4 injected ip in 3 divided doses daily at 8-hr intervals	4.2 ± 0.17 [1]	0.85 ± 0.17	10.67 ± 0.14	12.26 ± 0.34	0.45 ± 0.04
2 —Mg + 10 μg (D-T_4 injected ip in 3 divided doses daily at 8-hr intervals)	5.4 ± 0.10	0.72 ± 0.04	10.22 ± 0.10	10.02 ± 0.44	1.15 ± 0.49
3 —Mg + 30 μg (D-T_4 injected ip in 3 divided doses daily at 8-hr intervals)	4.4 ± 0.25	0.70 ± 0.07	9.98 ± 0.19	11.21 ± 0.57	0.81 ± 0.19
4 —Mg + 50 μg (D-T_4 injected ip in 3 divided doses daily at 8-hr intervals)	4.5 ± 0.27	0.63 ± 0.05	9.81 ± 0.10	11.33 ± 0.19	0.41 ± 0.01
5 —Mg + 10 μg (D-T_4 injected ip in 5 divided doses daily at 4-hr intervals)	4.8 ± 0.14	0.66 ± 0.06	10.37 ± 0.03	11.18 ± 0.44	0.51 ± 0.09
6 —Mg + 30 μg (D-T_4 injected ip in 5 divided doses daily at 4-hr intervals)	4.7 ± 0.21	0.77 ± 0.07	10.22 ± 0.17	11.38 ± 0.45	0.53 ± 0.11
7 —Mg alkalinized water injected ip 3 times daily at 8-hr intervals	5.5 ± 0.23	0.69 ± 0.05	10.42 ± 0.17	9.63 ± 0.17	12.11 ± 3.46

[1] Mean ± SEM (5 animals per treatment, except group 7 which had 10). All rats fed 15 g of a magnesium-deficient diet (4.9 ppm) during the 4-day experimental period.

viously cited evidence of a lesser calorigenic effect of D-T_4 compared to L-T_4.

D-Thyroxine has been shown to be absorbed as readily as L-T_4 from loops of rat intestine (9). Sung and Middlesworth (10), using washed loops of rat intestines, reported that less than 35% of an L-T_4 dose was absorbed in 4 hours and that this was independent of the dose given over a 100-fold range (0.3 to 30 μg). Thus the animals in the present studies fed 10 to 14 g of a diet containing 10 ppm D- or L-T_4 were obtaining approximately 35 to 50 μg of the hormones.

Kidney calcification was prevented when the D-isomer was given intraperitoneally at 3- to 8-hour intervals. L-Thyroxine was effective at all levels tested, independent of the frequency or route of administration. Thus, D-T_4 can prevent calcium deposition in kidneys of magnesium-deficient rats without producing as severe a growth reduction as that associated with L-T_4 administration. This is a situation directionally similar to that of its equivalent hypocholesterolemic effects without so great a calorigenic effect in humans (5).

These observations on D-T_4 demonstrate that the effectiveness of its action is determined by the frequency of administration. This could be explained in part by the shorter half-time of 14 hours reported for this isomer (6). When injected at the shorter intervals or in the diet, D-T_4 was capable of exerting a calorigenic effect and of preventing kidney calcification comparable to that of the L-isomer.

In making these comparisons between L- and D-T_4 it is important to make a distinction between total activity and speed or extent of action at a given time. Tata (11) suggested that a difference between total activity and speed of action can be made only for reversible reactions such as basal metabolic rate or serum cholesterol levels. In the case of irreversible actions like metamorphosis and body growth, the hormone which reaches the receptor sites sooner or in relatively larger quantities will be the first to initiate a series of one-way reactions. In this study, kidney calcification being an irreversible process (12), the D-isomer was unable to exert its protective effect once the Ca accumulation had commenced.

It is also possible that D-T_4, being less tightly bound to the thyroxine-binding serum protein than L-T_4, can diffuse more easily from blood to tissues. Thus the decreased binding affinity of D-T_4, coupled with its shorter half-time, allows the hormone to exert its inhibitory effect at the kidney tissue level and to be metabolized in a shorter period of time than L-T_4. Continuous administration of D-T_4 permits it to be present in amounts which will be available

both for degradation and for prevention of kidney calcification.

Although processes regulating the distribution and metabolism of D- and L-T_4 indirectly affect their action, the site and mode of action of these hormones in inhibiting renal calcification remain unclear. No single mechanism of action has been proposed to explain this effect, but it is likely that T_4 exerts its protective action on the kidney by a primary action on kidney mitochondria. The mitochondrion has been suggested as the primary locus of thyroid hormonal action (13).

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Metabolites of Vitamin D_3 and Their Biologic Activity [1,2]

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ABSTRACT The metabolism of vitamin D_3 has been studied after intravenous injection of 10 IU [1, 2-^{3}H]-vitamin D_3 to vitamin D-deficient rats. A new chromatographic system achieved the separation of unchanged vitamin D and 11 radioactive metabolites of which only one (25-hydroxycholecalciferol) exhibits intense antirachitic activity. An important proportion of the radioactivity in bones and small intestine is represented by this active metabolite. By way of contrast, liver and kidneys accumulate selectively unchanged D_3. Plasma as expected contains large amounts of 25-hydroxycholecalciferol.

An elucidation of the mechanism of action of vitamin D requires an understanding of its pathway of metabolism including the identification of its products and intermediates (1). Kodicek (2) was the first to report biologically inactive "breakdown products" of vitamin D all of which remain unidentified. Work done in our laboratory demonstrated clearly the presence of at least three metabolic fractions (peaks I, II and IV) derived from vitamins D_2, D_3 and D_4 in lipid extracts of blood and various tissues of vitamin D-deficient rats (1, 3–6). Of these, peak I has been positively identified as an ester of vitamin D and long-chain fatty acids (5). Peak II still remains unidentified but is of minor significance. Peak IV, however, proved to be of great interest. Not only was it as effective as vitamin D itself in curing rickets but it acted more rapidly than vitamin D in stimulating intestinal transport of calcium. Thus, it was thought to contain the metabolically active form of the vitamin (1, 4, 7). This fraction, however, appeared to be heterogeneous, composed of more than one metabolite.[3] Hence, it was necessary to develop a chromatographic system which would resolve these components. It is the purpose of this report to describe such a procedure which separates peak IV into six components. Of these six, only one retains the ability to cure rickets in rats, which probably represents the metabolically active form of vitamin D. The new chromatographic system presented here achieves the separation of 11 metabolites of the vitamin in the rat. This separation has now made possible the successful isolation and identification of 25-hydroxycholecalciferol, the metabolically active form of vitamin D (8, 9).

MATERIALS AND METHODS

Radioactive vitamin D_3. [1, 2-^{3}H]-Vitamin D_3, synthesized in this laboratory (10), was purified by chromatography on a column of silicic acid and stored in benzene solution. The purity of the radioactive preparation was assessed by ultraviolet spectrophotometry, biological assay (line-test) (11), and chromatographic behavior as previously described (10). The specific activity was 24,453 dpm/IU or 0.44 μCi/μg.. For intravenous injections, an appropriate amount of the benzene stock solution was dried under N_2 and redissolved in plasma from vitamin D-deficient rats to achieve a concentration of 200 IU/ml.

[4-^{14}C]-Vitamin D_3 [4] was purified by silicic acid chromatography (10) and stored in benzene at 4°. Proper dilution with non-radioactive crystalline vitamin D_3 [5] reduced the specific activity to 51.8 dpm/IU or 9.33×10^{-4} μCi/μg. The intravenous doses were prepared by removing the solvent under nitrogen and redissolving the vitamin D_3 in absolute ethanol at a concentration of 200,000 IU/ml.

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[2] Supported by Public Health Service Research Grant no. AMO 5800-07 and by the Harry Steenbock fund of the Wisconsin Alumni Research Foundation.

[3] Neville, P. F., J. Lund, H. Morii and H. F. DeLuca 1967 Biologically active metabolites of vitamin D. Federation Proc., 26: 697 (abstract).

[4] Purchased from Philips-Duphar, Weesp, The Netherlands.

[5] Cholecalciferol, USP XVII, Philips-Duphar.

Animals. Male, weanling rats of the Holtzman strain, housed in individual hanging wire cages were fed the vitamin D-free diet no. 11 (0.4% Ca, 0.3% P) of Guroff et al. (12) for 3 to 5 weeks. At this time the rats were vitamin D-deficient but not rachitic, and weighed an average of 150 g. Throughout the experimental period, they were fed the same diet.

Extraction of tissues. Extractions were carried out with a mixture of chloroform–methanol–water according to the procedure of Bligh and Dyer (13) modified in this laboratory (4). The chloroform extracts were evaporated to dryness in a flash evaporator under water-pump vacuum at 35° and redissolved in 5 or 10 ml redistilled (67°) petroleum ether (Skellysolve-B) before chromatography. Three aliquots of lipid extract in Skellysolve-B were pipetted into counting vials for measurement of the chloroform-soluble radioactivity. The aqueous-soluble phase and nonextractable precipitates were separately collected for radioactivity measurements. Before counting, the precipitates were washed three times with distilled water which was added to the aqueous-soluble phases (fig. 1).

Chromatography of vitamin D metabolites. Tissues and plasma extracts in redistilled Skellysolve-B solution were subjected to column chromatography on silicic acid. Prior to use, silicic acid, 100 mesh,[6] was treated according to Hirsch and Ahrens (14). Glass columns cooled to 10° by a water jacket were filled with a slurry of 35 g silicic acid in Skellysolve-B. Packed under a pressure of 3 psi, the adsorbent formed a column of 50 cm by 1.5 cm which gave a flow rate of approximately 1.5 ml/minute. Samples were applied to the column in 5 to 10 ml Skellysolve-B, followed by a 5-ml Skellysolve-B rinse. Adsorbed samples were then eluted with the following solvent system in the order given: 1) a hyperbolic gradient zero to 50% diethyl ether in Skellysolve-B was obtained by superimposing a holding chamber containing 250 ml of 75% diethyl ether in petroleum ether on a constant volume Erlenmeyer mixing chamber containing 230 ml of 100% petroleum ether (15); 2) a hyperbolic gradient 50 to 94% diethyl ether in Skellysolve-B was achieved by adding to the empty holding chamber 400 ml of 100% diethyl ether; 3) a hyperbolic gradient from 6% Skellysolve-B, 94% ether to 1.6% Skellysolve-B, 94.8% ether, 3.6% absolute methanol was obtained by adding 300 ml of 5% methanol in diethyl ether to the empty holding chamber; 4) a hyperbolic gradient from 1.6% Skellysolve-B, 94.8% ether, 3.6% methanol to 0.7% Skellysolve-B, 68.7% ether, 30.6% methanol was then utilized by adding 200 ml of 50% methanol in diethyl ether to

[6] Mallinckrodt Chemical Works, St. Louis, Mo.

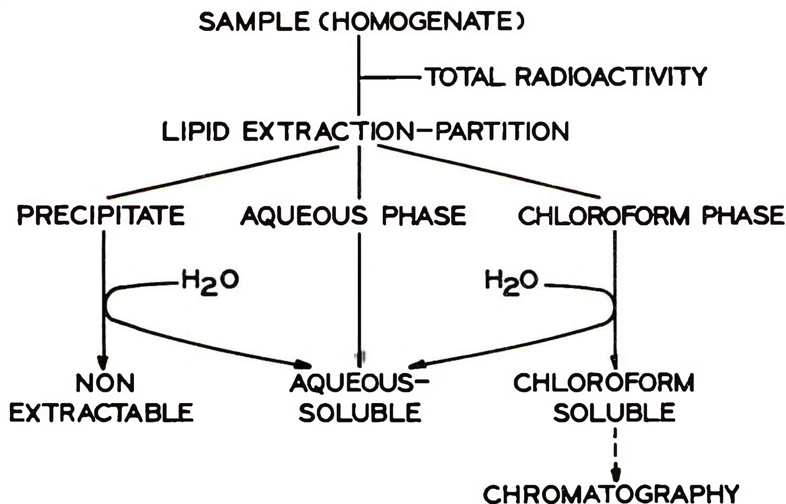

Fig 1 Flow-sheet of tissue radioactivity measurements.

the empty holding chamber; and 5) finally, an addition of 200 ml absolute methanol directly applied on the column without the intermediate mixing chamber concluded the elution.

The effluents were collected as 10-ml fractions directly in glass counting vials for radioactivity measurements. When biological assays were performed, 10-ml fractions were collected in test tubes and 0.5 ml was transferred to glass counting vials for radioactivity measurements while the remaining 9.5 ml was kept for bioassays.

Biological assays. After the radioactivity profile had been determined, the fractions from each peak were pooled; the solvent was removed with a flash evaporator and the residue redissolved in a small volume of diethyl ether. On the basis of their measured radioactivity and the specific activity of the parent ^{14}C-vitamin D_3 (51.8 dpm/IU), a calculated amount of cottonseed oil [7] was added, and the ether removed with nitrogen giving a solution containing the equivalent of 40 IU vitamin D_3/ml oil. Until used for bioassays, the oily preparations were kept frozen at $-10°$.

The newly isolated metabolites were assayed for their antirachitic activity in the rat according to official USP procedures (11) (line-test). The rats were dosed with the equivalent of 4 IU vitamin D in 0.1 ml oil by gastric tube, on the assumption that the specific activity of each metabolite is equal to that of the parent ^{14}C-vitamin D_3. In addition, positive reference rats were fed 4 IU standard vitamin D_3 in 0.1 ml cottonseed oil, and negative controls received 0.1 ml pure cottonseed oil alone.

Radioactivity measurements. The fractions were dried in an air stream under an infrared lamp and counted in a toluene counting solution consisting of 2.0 g PPO and 100 mg dimethyl POPOP/liter toluene AR.

The "dose-standards" were measured by delivering into counting vials in triplicate the same dose of labeled vitamin D as was injected into the rats. [1, 2-^{3}H]-Vitamin D_3, 0.05 ml in plasma was evaporated to dryness and dissolved in 0.5 ml Nuclear Counting Solution reagent [8] (16) before adding the toluene counting solution.

The radioactivity in each lipid extract submitted to chromatography was also determined and is referred to as chloroform-soluble radioactivity. Aliquots of the aqueous phases from each extraction were dried in counting vials, redissolved in a few drops of distilled water and 2 ml N.C.S. reagent [9] and finally mixed with toluene-counting solution. This gave the aqueous-soluble radioactivity. The nonextractable radioactivity was obtained by dissolving the washed precipitate in N.C.S. reagent, and counting in toluene-counting solution. To determine the total radioactivity in different organs, tissue samples in triplicate were combusted according to the procedure of Kelly et al. (17), using a Thomas Ogg safety igniter.[10] Weighed liver or kidney slices and small intestinal segments were inserted in cellophane combustion envelopes [11] and lyophilized before combustion. Whole femurs were weighed, split to remove the bone marrow, and dried by lyophilization in preparation for combustion. The tritiated water resulting from the combustion was dissolved and counted in combustion-counting solution consisting of 6 g PPO, 50 mg dimethyl POPOP, 200 ml absolute ethanol, and toluene AR to 1:1 (10). Alternatively, total radioactivity in tissues was calculated as the sum of the chloroform-soluble, aqueous-soluble and nonextractable radioactivity.

Total plasma radioactivity was determined in triplicate on samples dissolved in N.C.S. reagent [12] and mixed with toluene-counting solution. All radioactivity measurements were performed with a liquid scintillation counter [13] equipped with an automatic external standardization system 3951 (18). Counting efficiencies for ^{3}H were of the order of 25 to 35% with the toluene-counting solution, and 15 to 25% with the combustion-counting solution.

Experimental procedure. Ten international units [1,2-^{3}H]-vitamin D_3 in 0.05 ml plasma solution were injected intravenously, via the jugular vein, to eight vita-

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[7] Wesson Oil, Wesson Sales Company, Fullerton, Calif.
[8] Nuclear-Chicago Corporation, Des Plaines, Ill.
[9] See footnote 8.
[10] A. H. Thomas Company, Philadelphia, Penna.
[11] Ivers-Lee Company, N. J.
[12] See footnote 8.
[13] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

min D-deficient rats under light ether anesthesia. After 4 and 24 hours, respectively, two groups of four rats each were exsanguinated by cardiac puncture under ether anesthesia. The plasma was separated from the heparinized blood by centrifugation within 2 hours and its total radioactivity was determined. The radioactivity in the total blood plasma was calculated from an estimated plasma volume equivalent to 3% of body weight. Storage of the blood plasma as well as of any tissue was done at −10° until chromatography.

Liver and kidneys were removed by dissection, rinsed in 0.9% NaCl, blotted on absorbent paper, and weighed before being frozen until extraction.

The long bones (femurs, tibias, humeri, ulnas and radii) were dissected from adhering muscles and ligaments; they were weighed, split to remove the bone marrow, and frozen until they could be extracted.

The small intestine was removed, dissected from the mesentery, cut into segments which were gently squeezed with forceps to expel the intestinal contents, and rinsed with 0.9% NaCl before being frozen.

To collect large amounts of metabolites for bioassays, 10,000 IU of [4-^{14}C]-vitamin D_3 in 0.05 ml absolute ethanol were injected intravenously via the jugular vein to 10 vitamin D-deficient rats. After 18 hours, the animals were exsanguinated through cardiac puncture under ether anesthesia. Plasma and tissues were prepared, extracted and chromatographed as decribed above. The isolated radioactive fractions were subsequently tested for their biopotency, as described under biological assays.

RESULTS

As shown by its chromatographic pattern (fig. 2), the labeled [1, 2-^{3}H]-vitamin D_3 dissolved in plasma was free of any polar contaminants, although it contained a small amount (3.6%) of less polar material. This represents presumably some previtamin D_3 in thermal equilibrium with the vitamin (19). Nevertheless, the ultraviolet spectrum and the biological activity determined by the line-test assay were consistent with a specific activity of 24,453 dpm/IU.

The distribution of the radioactivity in plasma and tissues after intravenous injection of 10 IU [1, 2-^{3}H]-vitamin D_3 to vitamin D-deficient rats is given in table 1 and 2. Four hours after the dose (table 1), the liver had accumulated more radioactivity than the blood or any other tissue (34.2%). Following in decreasing order are the plasma compartment (20.6%), the skele-

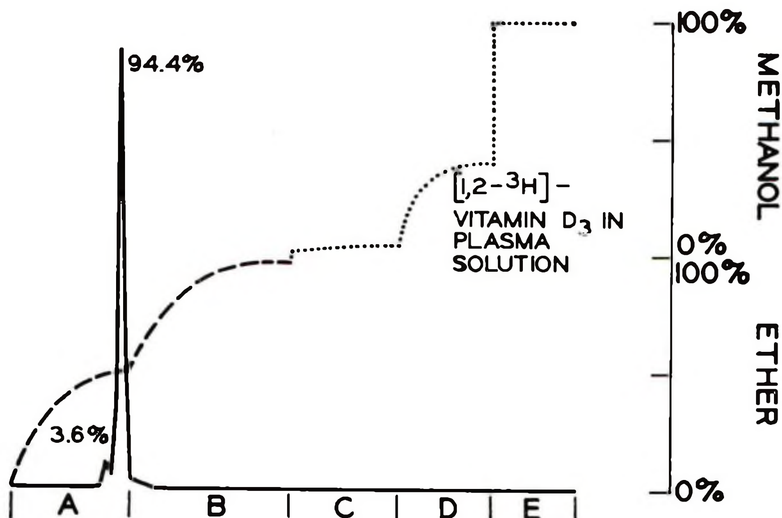

Fig. 2 Silicic acid column profile of radioactivity from [1, 2-^{3}H]-vitamin D_3 dissolved in D-deficient plasma (dose).

TABLE 1

Total radioactivity in plasma and tissues 4 hours after intravenous injection of 0.25 μg [1,2-³H]-vitamin D₃ to vitamin D-deficient rats

	Plasma		Small intestine		Skeleton		Liver		Kidneys	
	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose
	%	%	%	%	%	%	%	%	%	%
Chloroform soluble	99.6 [1]	20.5	85.4	2.1	85.4	10.9	99.1	33.9	99.0	7.8
Aqueous soluble	0.3	0.07	12.0	0.3	13.9	1.8	0.5	0.2	0.7	0.05
Nonextractable	0.05	0.01	2.6	0.06	0.7	0.1	0.4	0.1	0.3	0.02
Total radioactivity	100	20.6 ±0.88(4) [2]	100	2.5	100	12.8 ±0.84(4)	100	34.2	100	7.9

[1] Single data represent values for pooled samples from four rats. SEM are given for separate measurements.
[2] Mean ± SEM (number of rats).

ton (12.8%), the kidneys (7.9%), and the small intestine (2.5%). Practically all the radioactivity in plasma, liver and kidneys (over 99%) was lipid soluble, and partitioned in the chloroform phase during the extraction. In contrast, a significant amount of water-soluble radioactive material was present in the small intestine (12.0%) and the bones (13.9%). Almost no radioactivity remained unextracted except a small fraction (2.6%) in the case of the small intestine.

Twenty-four hours after dosage (table 2), plasma and liver radioactivities had fallen to 10.6 and 14.9%, respectively, while the skeleton (16.0%) and kidneys (18.8%) had gained a significant amount of the dose. There was a very small decrease in the small-intestine radioactivity (1.8%). Though virtually all the plasma radioactivity was still chloroform soluble, an increasing portion (4 to 8%) of the tissue radioactivity remained unextracted from the precipitates.

The "relative incorporation," expressed as the fraction of the dose accumulated in 1 g dry tissue, is an indication of the tissue concentration of vitamin D and its metabolites (table 3). Four hours after dosage, there was a striking concentration of label both in liver (20.8% of dose/g dry tissue), and kidneys (24.8%), compared with the low concentrations in small intestine (3.4%) and bone (1.2%). While the relative incorporation did not change significantly at 24 hours in the small intestine (3.7%) and bones (2.2%), it decreased in the liver (13.7%), but increased further in the kidneys to reach the very high concentration of 81.3%/g dry matter.

In highly heterogeneous tissues, such as bone and small intestine, a correction must be made to express the true relative incorporation of dose in the cells. As in a previous work (10), bone cells are estimated as 4% of the dry weight of undecalcified bone, and would have a true relative incorporation of 30 and 55% of dose/g dry tissue after 4 and 24 hours, respectively. The small intestine is assumed to be made of 30% mucosa and 70% muscle and connective tissue. It has already been demonstrated that muscle has a very low relative incorporation decreasing slowly as time

TABLE 2

Total radioactivity in plasma and tissues 24 hours after intravenous injection of 0.25 μg [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

	Plasma		Small intestine		Skeleton		Liver		Kidneys	
	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose	Sample radio-activity	Dose
	%	%	%	%	%	%	%	%	%	%
Chloroform soluble	99.1 [1]	10.5	83.5	1.5	90.6	14.5	87.2	13.0	93.3	17.5
Aqueous soluble	0.9	0.09	7.6	0.1	5.2	0.8	4.9	0.7	1.2	0.2
Nonextractable			8.9	0.2	4.2	0.7	7.9	1.2	5.6	1.1
Total radioactivity	100	10.6 ±0.67(4) [2]	100	1.8	100	16.0 ±0.97(4)	100	14.9 ±1.8(4)	100	18.8 ±1.1(4)

[1] Single data represent values for pooled samples from four rats. SEM are given for separate measurements.
[2] Mean ± SEM (number of rats).

elapses after dose (10). One can roughly estimate that the mucosal cells accumulate about 10.9 and 12% of dose/g of dry tissue after 4 and 24 hours, respectively.

The chloroform phases of plasma and tissue extracts, obtained 4 and 24 hours after dose, have been chromatographed as described in Methods. Figure 3 illustrates a characteristic chromatographic pattern obtained from the plasma 24 hours after dose. All the chromatographic patterns are similar except for the relative proportion of radioactive peaks, which are given at 4 hours (table 4) and 24 hours (table 5) after dose. Twelve radioactive fractions were consistently observed, although some of them were incompletely separated (Va and Vb, VIa and VIb), or represented only minimal amounts (IIa, IVa, VIb, and VIIb). In such instances, their combined amounts were reported.

Fractions I, II and III had already been separated in our laboratory, using a chromatographic technique similar to the present one in its starting solvent system (3–5). Fractions I and III have been identified as vitamin D esters and unchanged vitamin D, respectively, while fraction II has not yet been characterized. The newly isolated fraction IIa was previously included either in fraction I or II. Its composition is not known. The so-called "peak IV" active metabolite of vitamin D previously discovered by the same authors has now been resolved into six sub-fractions IVa, IV, Va and Vb, VIa and VIb, by means of a refined gradient system. Fractions IVa and IV were eluted at a concentration of about 90% ether in Skellysolve-B. Fractions Va and Vb were eluted by a very small concentration of approximately 3% methanol in ether (95%)–Skellysolve-B (2%), while VIa and VIb came out with 25% methanol in ether (74%)–Skellysolve B (1%). As shown later, the biological activity was specific to the purified fraction IV. These fractions have not been characterized, except the biologically active "peak IV" which has been most recently identified as 25-hydroxycholecalciferol in our laboratory (8, 9).

Fractions VIIa and VIIb were eluted by 100% methanol, and were probably not brought off the column with the solvent

TABLE 3

Relative incorporation of dose in tissues 4 and 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Time interval	Small intestine	Skeleton	Liver	Kidneys
hr		*% dose/g dry tissue*		
4	3.4 [1] 10.9 [3]	1.2±0.6(4) [2] 30.0 [3]	20.8	24.8
24	3.7 12.0 [3]	2.2±0.2(4) 55.0 [3]	13.7±1.7(4)	81.3±5.6(4)

[1] Single data represent values for pooled samples from four rats. SEM are given for separate measurements.
[2] Mean ± SEM (number of rats).
[3] Corrected values for small intestine mucosal cells and bone cells (see text).

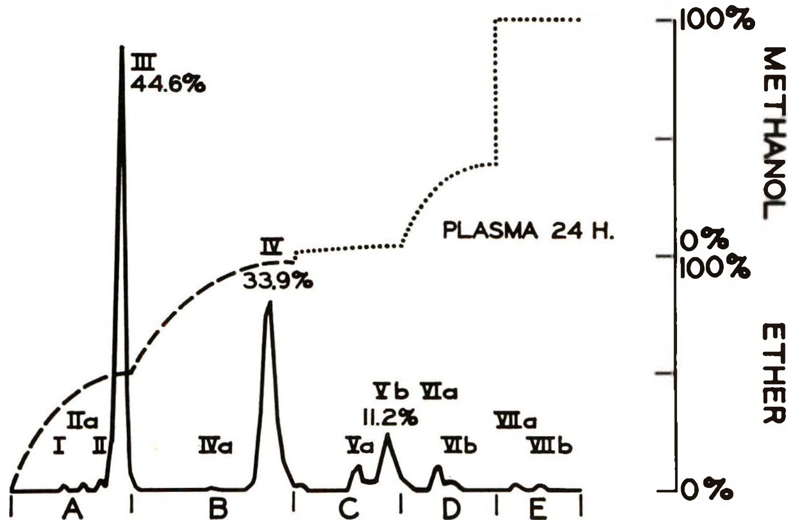

Fig. 3 Silicic acid column profile of radioactivity from pooled lipid extracts of plasma obtained 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to four vitamin D-deficient rats.

TABLE 4

Chromatographic fractions of plasma and tissues extracts 4 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.3 [1]	1.5	1.8	1.1	1.2
IIa	0.2	0.4	0.3	1.1	0.8
II	1.9	3.0	5.0	3.1	3.2
III	73.1	62.2	75.7	88.8	76.6
IVa	—	1.6	1.4	—	1.0
IV	16.0	14.1	9.0	1.7	3.9
Va	1.6	2.0	1.0	0.6	2.6
Vb	2.6	2.8	2.3	0.6	2.4
VIa	0.9	4.0	0.9	0.7	3.1
VIb	0.4	0.5	0.4		
VIIa	0.7	2.5	0.4	0.3	1.9
VIIb	0.3	0.7	0.1	—	0.3

[1] All values are percent of total chromatographed radioactivity from pooled samples of four rats.

TABLE 5

Chromatographic fractions of plasma and tissues extracts 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.5 [1]	4.4	1.5	2.8	2.7
IIa	0.6	2.1	2.0	1.5	0.8
II	0.8	4.0	4.2	3.2	4.0
III	44.6	38.8	53.5	75.9	73.6
IVa	0.4	—	2.1	—	—
IV	33.9	16.9	16.0	10.6	8.1
Va	2.5	16.3	9.0	2.5	1.8
Vb	11.2				
VIa	2.0	2.2	2.5	—	1.8
VIb	0.8				
VIIa	0.3	2.9	2.2	1.1	3.3
VIIb	0.3	2.4	1.7	0.4	2.4

[1] All values are percent of total chromatographed radioactivity from pooled samples of four rats.

gradient used previously (3, 4) which did not produce over 87% methanol concentration in the effluent. These fractions have not been identified. Further stepwise elution with 150 ml water–methanol (10:90) did not bring any more radioactivity from the column from any of the extracts.

Overall recoveries of radioactivity from chromatographed material varied between 95 and 102%.

The four major fractions (III, IV, Va and Vb) usually represent over 80% of the chromatographed radioactive material. It is evident that 24 hours as well as 4 hours after dose, the major radioactive fraction in plasma and all tissues is the "peak III," unchanged vitamin D_3. This fraction decreased significantly in all cases, between 4 and 24 hours after dose. Concomitantly, there was a relative increase in the proportion of peaks IV and Va + b (tables 4 and 5). It should be noted that plasma, small intestine and skeleton contained the highest proportion of peak IV and Va + b both 4 and 24 hours after dosage. The minor fractions, I, IIa, II, VIa +b, and VIIa + b, tended to increase in all samples, from 4 to 24 hours after dosage. The relative proportion of these substances was higher in all tissues than in plasma.

Tables 6 and 7 give the absolute amounts of fractions I, II, III, IV and Va + b in plasma and tissues 4 and 24 hours after dose, respectively. Four hours after dose, the liver had accumulated the largest fraction of the unchanged vitamin D_3, 30.1% of the injected dose (table 6). The remainder of the unchanged vitamin D_3 was distributed mostly in the plasma compartment (15.0% of dose), skeleton (8.2%) and kidneys (6.0%). The small intestine, owing to its very small accumulation of dose, contained only a small fraction of the vitamin D and metabolites. Most of the "peak IV" metabolite was present in the plasma, where it represented 3.3% of the dose. Fractions I, II and Va + b were less than 1% of the dose in any of the tissues or plasma examined. Twenty-four hours after an intravenous dose (table 7) the amount of unchanged vitamin D had decreased considerably in the plasma (17.1 down to 4.7% of dose) and the liver (30.1 to 9.9% of dose), compared with the 4-hour period. It also decreased in small intestine (1.31 to 0.6%), but did not change much in skeleton. Unexpectedly, the kidneys doubled their content of vitamin D (6.0 to 12.9% of dose), and contained more vitamin than the plasma or any other tissue investigated.

The amount of "peak IV" metabolite increased slightly in plasma and all tissues except the small intestine. Plasma still contained the largest part of "peak IV" (3.6% of dose). There was no consistent change in the quantities of peak I and II. Fractions Va + b increased slightly in plasma (0.9 to 1.4% of dose), and skeleton (0.4 to 1.3%), but did not change in the small intestine, liver and kidneys.

The antirachitic activity of the vitamin D metabolites was assessed by the rat line-test method (11), and the results are sum-

TABLE 6

Amounts of vitamin D_3 and its major metabolites in plasma and tissues 4 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromato-graphic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.06 [1]	0.03	0.2	0.4	0.1
II	0.4	0.06	0.5	1.0	0.2
III	15.0	1.3	8.2	30.1	6.0
IV	3.3	0.3	1.0	0.6	0.3
Va+b	0.9	0.1	0.4	0.4	0.4

[1] All values are percent of administered dose from pooled samples of four rats.

TABLE 7

Amounts of vitamin D_3 and its major metabolites in plasma and tissues 24 hours after intravenous injection of 10 IU [1,2-^{3}H]-vitamin D_3 to vitamin D-deficient rats

Chromato-graphic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
I	0.05 [1]	0.07	0.2	0.4	0.5
II	0.08	0.06	0.6	0.4	0.7
III	4.7	0.6	7.8	9.9	12.9
IV	3.6	0.2	2.3	1.4	1.4
Va+b	1.4	0.2	1.3	0.3	0.3

[1] All values are percent of administered dose from pooled samples of four rats.

marized in table 8. As specified under Methods, these fractions were obtained from a pool of 10 rats injected with 10,000 IU [4-^{14}C]-vitamin D_3/rat, and killed 18 hours later. Positive reference rats, fed 0.1 μg crystalline vitamin D_3 in 0.1 ml cottonseed oil gave the required 40 IU/μg response whereas the negative controls fed 0.1 ml pure cottonseed oil showed no line-test.

Among the fractions assayed, only peak III (unchanged vitamin D_3) and peak IV gave consistently a response close to that of crystalline vitamin D_3. There was some variation in the response to peak IV material from various tissues, with plasma peak IV as the most active (125% of crystalline vitamin D_3 activity). Fractions IIa from liver and kidneys had some antirachitic activity (10 and 42%, respectively, of that of vitamin D_3). Peak IVa from liver also showed some activity (18% of vitamin D activity), but it was not possible to confirm it with other extracts. Peak Va + b from plasma and skeleton gave no response, but kidney peak Va + b was apparently active (37% of vitamin D activity). Fractions VIa + b were found inactive in plasma and liver. Fractions VIIa +b were inactive in plasma and bone, and showed questionable activity (less than 10% of vitamin D_3 activity) in liver extract.

DISCUSSION

Much attention has been paid in this laboratory to the study of a highly biologically active metabolite of vitamin D_3 in rats initially isolated as a peak IV fraction (3, 4). Further methodologic investigations provided indications that this fraction was nonhomogeneous [14]. We report here the successful resolution of the former "peak IV" fraction into six different subfractions: IVa, IV, Va, Vb, VIa, VIb. Only the newly purified metabolite IV exhibits intense biological activity. It was very recently identified by Blunt et al. (8, 9) as 25-hydroxycholecalciferol, and is thought to be the active principle of vitamin D_3. In addition, one low polarity fraction IIa, and two very polar chromatographic fractions VIIa and b, have been separated. The new chromatographic system achieved the separation of 11 different metabolic fractions instead of the 3 previously obtained from plasma and tissue extracts of rats injected with tritiated vitamin D_3. It should be noted

[14] See footnote 3.

TABLE 8

Antirachitic activity of chromatographic fractions of plasma and tissues extracts

Chromatographic fractions	Plasma	Small intestine	Skeleton	Liver	Kidneys
IIa	—	—	—	4 [1]	17
III	40	—	—	—	—
IVa	—	—	—	7	—
IV	50	34	30	34	41
Va+b	0	—	0	—	15
VIa+b	0	—	—	0	—
VIIa+b	0	—	0	3	—

[1] The biological activity is expressed in IU/μg.
The amount of each fraction was estimated on the basis of the specific activity of the parent [4-^{14}C] vitamin D_3, i.e., 2,072 dpm/μg (51.8 dpm/IU).

that some resolution of the impure "peak IV" material has also been achieved by means of a dichloromethane-ether or dichloroethane-ether solvent system [15] (20). That these fractions do not represent degradation products occurring during storage, extraction or chromatographic procedure, was demonstrated by their absence in extracts of ^{3}H-vitamin D_3 added to plasma and by their increase in amount as a function of time after dose.

Of these 12 fractions, only peaks I, III and IV have been identified so far as, respectively, vitamin D esters (5), unchanged vitamin D (3), and 25-hydroxycholecalciferol (8, 9).

As far as biological activity is concerned, only the unchanged vitamin D_3 fraction (peak III) and the 25-hydroxycholecalciferol (peak IV) showed consistently an antirachitic effect comparable to that of crystalline vitamin D_3. Pure 25-hydroxycholecalciferol exhibits 140% of the vitamin D_3 antirachitic activity (9). In our experiments, however, plasma peak IV did not show more than 125% of vitamin D_3 activity, and the tissue extracts were somewhat less active than vitamin D itself. It is possible that this discrepancy is due either to radioactive contaminants very closely related chemically but biologically inactive, or to nonradioactive substances interfering with the antirachitic activity of 25-hydroxycholecalciferol. One should also consider that chemical degradation is likely to affect, to a higher extent, the very small amounts (less than 1μg/pooled tissue extract) isolated in the present experiments than the milligram quantities on hand for the previous formal determinations of biological activity. In previous work, vitamin D esters (peak I) and unidentified peak II have been found partially active (3–5).

Unchanged vitamin D_3 which accumulated first in liver and plasma disappeared rapidly from 4 to 24 hours. During the same period, it disappeared more slowly from the small intestine and bones, while it doubled its concentration in the kidneys. Concomitant with the decrease of unchanged vitamin D_3 was a slight increase in the amount of 25-hydroxycholecalciferol and of the more polar metabolites, in most tissues and plasma. The largest quantities of 25-hydroxycholecalciferol were found in plasma and skeleton. In contrast, liver and kidneys had always the smallest proportions of 25-hydroxycholecalciferol. Incidentally, peaks I and II did not change significantly between 4 and 24 hours. They always represented small fractions of the radioactivity (less than 5%) and were present mostly in liver, skeleton and kidneys.

The distribution of the total radioactivity among the tissues examined revealed a striking accumulation of the dose in the kidneys 24 hours after the intravenous 10 IU [1, 2-^{3}H]-vitamin D_3. While the radioactivity disappeared rapidly from liver and plasma and slowly from small intestine between 4 and 24 hours, it tended to increase in skeleton and doubled in the kidneys. At 24 hours the kidneys contained a higher proportion of the dose than any other tissue. Most of this radioactivity was lipid soluble and represented unchanged vitamin D_3. This radioactive uptake by the kidneys is much higher than previously reported (10). Such a difference may be related to the injection vehicle used in the

[15] Philips-Duphar, personal communication.

former experiments, namely 0.1% Tween 20[16] in 0.9% NaCl. The higher accumulation of dose in our observations might be due to the persistence of higher plasma levels of radioactivity when the labeled vitamin D_3 is injected in a plasma solution (21). Another, more unlikely, possibility is that Tween 20 was interfering locally with the kidney uptake of ^{3}H-vitamin D_3. The present report of an important uptake of vitamin D_3 by the rat kidneys supports the concept of kidney as a target for vitamin D action, because it is not related to the very small urinary excretion of vitamin D and metabolites in rats (22). Kidneys, however, do differ from the well-known "target organs," bones and small intestine, by their slower accumulation of dose and the much higher proportion of unchanged vitamin D_3. This might indicate a delayed effect, storage, or possibly a different mechanism of action of the vitamin in the kidney than in bones and small intestine.

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[16] Obtained from Atlas Powder Company, Wilmington, Del.

Regional Renal Adenosine Triphosphate Metabolism in Thiamine Deficiency [1]

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ABSTRACT This study assesses the effect of progressive thiamine deficiency and its subsequent repletion on renal adenosine triphosphate (ATP) metabolism and the activity of pyruvate decarboxylase and transketolase. Since renal cortex is primarily dependent on fatty acid metabolism whereas renal medulla utilizes glucose, these areas were studied separately. The data show a substantial fall in both pyruvate decarboxylase and transketolase activity during thiamine deficiency, with cortex and medulla equally affected. The medullary ATP concentration fell progressively and significantly with increasing thiamine deficiency, while cortical ATP remained normal. With thiamine administration there was a rapid return to normal of medullary ATP which correlated much better with a rise in pyruvate decarboxylase than transketolase activity. Thiamine pyrophosphate in vitro also fully reversed the depressed pyruvate decarboxylase of thiamine-deficient kidney but had no effect on transketolase activity. Data were obtained which suggest that regional renal metabolic rate is not increased in thiamine deficiency. This study therefore documents a rapidly reversible fall in medullary ATP concentration with thiamine deficiency and suggests that this effect is related to depressed pyruvate decarboxylation and presumably decreased ATP synthesis via the TCA cycle. Consistent with this concept the renal cortex which subserves primarily on nonglucose substrates shows no decrease in ATP.

Thiamine pyrophosphate is the coenzyme for three major biochemical reactions in the body, transketolation and the decarboxylation for both pyruvate and α-ketoglutarate. It is established that α-ketoglutarate decarboxylase activity is decreased relatively little in thiamine deficiency presumably due to the tight binding of the thiamine to this apoenzyme (1). The respective importance and consequences of depressed transketolase and pyruvate decarboxylase activity [4] in various tissues in thiamine deficiency have not been ascertained (2).

It has been suggested for some time, however, that a low-thiamine induced decrease in pyruvate decarboxylation in critical brain areas and in heart may lead to a decreased formation of tricarboxylic acid (TCA) cycle intermediates resulting in a diminished synthesis of adenosine triphosphate (ATP), an important ultimate source of energy in these organs (3–6). Recent studies in our laboratory have documented a depression of pyruvate decarboxylase activity, amounting to about 30%, in the cerebellum and brain stem of thiamine-deficient rats with signs of central nervous system dysfunction; however, the ATP levels in these tissues were normal (2).

The aims of the present study were to assess renal energy (ATP) metabolism in thiamine deficiency and to correlate this with changes in kidney pyruvate decarboxylase activity. The kidney was chosen for investigation because: 1) in thiamine deficiency renal pyruvate decarboxylase activity is depressed twice as much as in brain (2, 7); 2) the hexose monophosphate shunt (in which transketolation participates prominently) is responsible for only a trivial part of normal renal metabolism (8, 9); and 3) a variety of ill-defined renal physiologic abnormalities have

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[4] This reaction is part of a complex enzymatic process, responsible for the conversion of pyruvate to acetyl coenzyme A, which is designated as pyruvic acid dehydrogenase.

been described in thiamine deficiency without detailed biochemical kidney analysis (10, 11). It is, furthermore, generally agreed that a high fat diet "protects" thiamine-deficient animals against the onset of neurologic signs (12, 13) presumably by providing acetyl CoA directly from fat, thus bypassing the need for pyruvate decarboxylation. Because renal cortex utilizes primarily fatty acids for its metabolism, whereas renal medulla subserves on glucose and thus should theoretically be more susceptible to low-thiamine induced decrease in glucose utilization (9), the two regions of the kidney were examined separately.

EXPERIMENTAL PROCEDURE

Female littermate rats of the Sprague-Dawley strain, weighing 60–70 g were placed individually in metabolic cages. One member of each pair was given daily 20 g of a special synthetic thiamine-deficient diet,[5] while the second animal (control) was pair-fed with the same diet supplemented with thiamine (control diet).[6] The daily food intake of the pair-fed control rat depended on the previous day's measured food consumption of the first animal. One gram of the control diet provides more than twice the minimum daily requirement of thiamine for rats (7) and the pair-fed controls, although partly starved toward the end of the experimental period, ate at least 1.5 g of the diet per day. A third group of female rats of equal weight was fed a regular laboratory diet ad libitum and served as the "normal" control group. All animals were given free access to water. The thiamine-deficient rats developed signs of neurologic dysfunction (ataxia, incoordination, drowsiness) at about 4.5 to 5 weeks of being fed the diet, while the pair-fed controls remained asymptomatic. An intraperitoneal injection of 10 to 20 μg of thiamine hydrochloride resulted in a reversal of the neurologic signs within 16 to 36 hours. The weight curve of these thiamine-deficient and pair-fed control rats has been given previously (2).

For kidney analysis the thiamine-deficient, pair-fed control and normal control rats were quickly anesthetized with ether and the kidneys removed. Renal cortical and medullary pyruvate decarboxylase and transketolase activity as well as protein concentrations were measured in fresh tissue dissected by hand into the respective parts as described by Lee et al. (9). The papilla was not analyzed. Regional renal lactate and ATP concentrations were determined on kidney rapidly frozen in liquid nitrogen and then dissected while frozen into cortex and medulla. Pyruvate decarboxylase activity was measured by the procedure of Dreyfus and Hauser (7), transketolase activity by the method of Dreyfus and Moniz (14), protein concentration according to Lowry et al. (15), lactate by the enzymatic procedure of Hohorst (16) and ATP by the luciferin-luciferase luminescence reaction (17). Blood transketolase and the thiamine pyrophosphate (TPP) effect in blood were measured by the technique of Dreyfus (18). The details and validation of these procedures have been described previously (2). Serum sodium, potassium and blood urea nitrogen were measured by conventional techniques.

The metabolic rates of thiamine-deficient and control cortex and medulla were calculated in terms of the effects of ischemia on renal energy reserves. In this procedure the ATP, phosphocreatine (PCr) and lactate concentrations are first determined in the rapidly frozen cortex and medulla of one kidney removed without blood loss. Subsequently, the other kidney is removed and after 120 seconds of ischemia, it is frozen and the change in these compounds (as compared to those of the immediately frozen kidney) is then determined in the appropriate areas. In the ischemic kidney the rise in tissue lactate (Δlactate) is taken as an index of anaerobic utilization of endogenous glucose and glycogen, whereas the fall in PCr (ΔPCr) and ATP (ΔATP)

[5] Thiamine-deficient diet: sucrose, 68%; vitamin-free casein, 18%; vegetable oil, 10%; salt mixture USP XIV, 4%; vitamins: (in g/100 lb. diet) vitamin A, 4.5 (200,000 IU/g); vitamin D concentrate, 9.25 (400,000 IU); alpha-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; calcium pantothenate, 3.0; and: (in mg/100 lb. diet) biotin, 20; folic acid, 90; vitamin B_{12}, 1.35.

[6] Control diet in addition to the above contained thiamine hydrochloride, 1 g/100 lb. diet. Both diets were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

is representative of the utilization of the preischemic energy reserves. Accordingly, if lesser intermediates are ignored the renal metabolic rate, expressed in terms of change in high energy phosphates ($\Delta \sim P$) can be calculated as $2\Delta ATP + \Delta PCr + \Delta lactate$. The rationale for this procedure and derivation of the above formula are presented in detail elsewhere (19–21). The technique, while indirect and approximate, is believed to give a reasonable index of tissue metabolic rate and has been used to good advantage in normal kidney (20) and in brain where it has correlated predictably with the neurologic state (21). Inasmuch as PCr comprises a very small part of renal energy reserves (0.2 and 0.3 μmole/gram in the medulla and cortex, respectively), it was not considered in these calculations.

Statistical analysis was carried out by nonparametric techniques which do not assume a normal distribution. Where applicable the Wilcoxon matched pair test was employed (22).

RESULTS

The mean blood transketolase and TPP effect in 14 sets of thiamine-deficient rats with overt neurologic signs (4.5 to 5 weeks on thiamine-deficient diet) was 61.8 μg of sedoheptulose generated/milliliter whole blood/30 minutes and 43.9%, respectively, while these values in the pair-fed asymptomatic controls were 497.9 μg and 6.4%, and in 5 normal rats 866.6 μg and 4.2%. The blood transketolase level in the thiamine-deficient symptomatic rats is depressed by 87.6% ($P < 0.001$) as compared to the pair-fed controls. The whole brain total thiamine concentration in the symptomatic animals was also depressed to 15.7% of values in pair-fed controls ($P < 0.01$) (2). Blood transketolase in the pair-fed controls was significantly below that of normally fed controls while the blood TPP effect and brain thiamine concentrations in both groups were equal.

As shown in table 1, pyruvate decarboxylase and transketolase activity in both renal cortex and medulla of the symptomatic thiamine-deficient rats was substantially and significantly depressed as compared to pair-fed controls. Furthermore, the degree of depression of each enzyme in cortex and medulla was comparable. Contrariwise, the ATP level in renal cortex was normal whereas that in the medulla of the thiamine-deficient animals fell significantly. Pyruvate decarboxylase activity in kidneys of pair-fed controls was slightly (though not significantly) below values in normal controls. Cortical transketolase activity in pair-fed control

TABLE 1

Effect of thiamine deficiency on regional renal metabolism [1]

	Pyruvate decarboxylase [2]		Transketolase [3]		ATP [4]	
Cortex						
Normal control	289.0 ± 28.4		54.71 ± 4.27		1.57 ± 0.04	
Pair-fed control	225.5 ± 24.0		41.39 ± 4.22		1.73 ± 0.04	
Thiamine-deficient	68.1 ± 9.38		5.22 ± 0.23		1.71 ± 0.07	
Decrease	69.8%	$P < 0.005$	86.9%	$P < 0.04$	1.2%	(ns)
Medulla						
Normal control	164.2 ± 17.61		22.01 ± 1.05		1.72 ± 0.04	
Pair-fed control	116.3 ± 9.35		22.68 ± 1.69		1.66 ± 0.05	
Thiamine deficient	32.2 ± 5.69		2.71 ± 0.23		1.12 ± 0.06	
Decrease	72.3%	$P < 0.005$	87.5%	$P < 0.005$	32.5%	$P < 0.001$

[1] The thiamine-deficient rats exhibited neurologic signs (2) at the time of assay. The values in thiamine-deficient rats are statistically compared to those obtained in controls pair-fed for the same time, 4.5 to 5 weeks.

[2] Pyruvate decarboxylase activity is expressed as μmoles of pyruvate decarboxylated/45 min per gram kidney cortex or medulla, wet weight. The data are given as the mean ± SE of 8 determinations, each in triplicate.

[3] Transketolase activity is expressed as μmoles sedoheptulose elaborated/45 min per gram kidney cortex or medulla, wet weight. The data refer to the mean ± SE of 5 to 9 determinations, each in triplicate.

[4] ATP is given as μmoles/gram kidney cortex or medulla wet weight. The data refer to the mean ± SE of 10 to 12 determinations.

rats was significantly ($P < 0.01$) below normal control data.

The progressive effect of increasing thiamine deficiency on medullary pyruvate decarboxylase and transketolase activity as well as on medullary ATP concentration and medullary weight is shown in figure 1. These data indicate that at 3 and 5 weeks of thiamine deficiency the medullary transketolase was depressed by 86.6 and 87.5%, and pyruvate decarboxylase activity by 66.0 and 72.1%, respectively. Medullary ATP concentration fell progressively with increasing thiamine deficiency, decreasing by 9, 18.2, 24.6 and 32.5% at 2, 3, 4 and 5 weeks, respectively ($P = 0.025$ or less). Within 24 hours of injection of 10 to 20 µg of thiamine hydrochloride, the neurologic signs were reversed, the medullary ATP concentration became normal, medullary pyruvate decarboxylase activity rose to a level only 29% below control values, but medullary transketolase, although slightly higher ($P < 0.03$), was still depressed by 79.6%. After three daily doses of thiamine, medullary ATP remained normal, pyruvate decarboxylase activity was 42% below normal, but transketolase was still

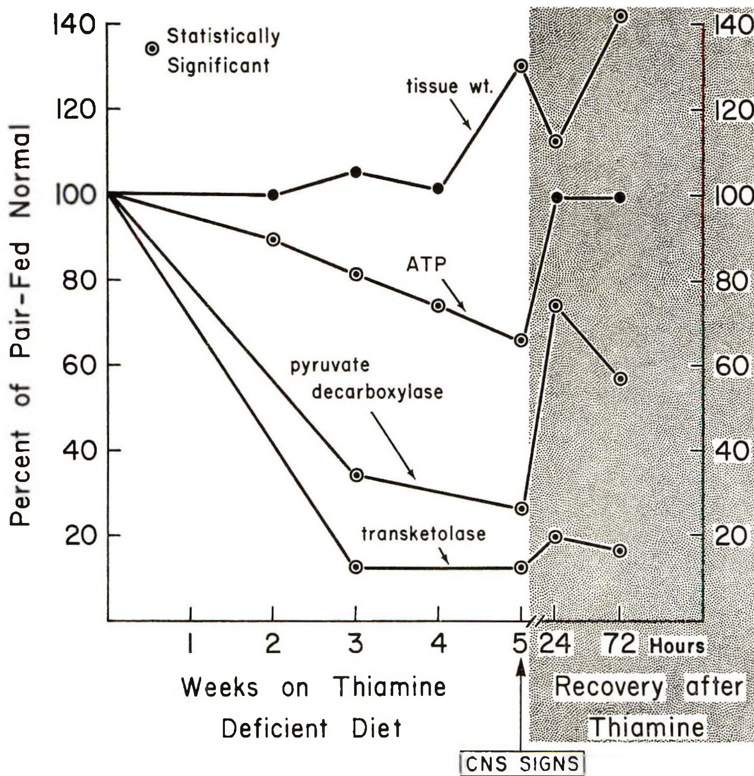

Fig. 1 The effect of progressive thiamine deficiency and its repletion on renal medullary weight, its ATP concentration and the activity of pyruvate decarboxylase and transketolase. Each point is the mean of 6 to 10 thiamine-deficient rats. The data are expressed as a percentage of values obtained in pair-fed control animals killed at the same time. Tissue weight refers to total medullary weight of thiamine-deficient rats initially expressed in percent of their body weight and then compared to the same values in pair-fed controls. Results which significance refers to a P value < 0.05, determined as indicated in the Experimental Procesignificance refers to a P value < 0.05, determined as indicated in the Experiment Procedure. Note that the time scale during recovery from thiamine deficiency is somewhat extended for ease of reproducibility.

depressed by 83%. In addition, blood transketolase after both one and three daily injections of thiamine, failed to increase over values obtained in the symptomatic animals and the TPP effect remained increased, 53.8 and 25.0%, respectively ($P > 0.05$). These data not only define the progressive decrease in ATP concentration in renal medulla with increasing thiamine deficiency but suggest that the reconstitution of a normal ATP level correlates better with a rise in medullary pyruvate decarboxylase than transketolase activity.

Medullary weight as percentage of body weight at 2, 3 and 4 weeks of thiamine deficiency was comparable to that of pair-fed controls but incerased by 30% at 5 weeks and remained heavier by 13.1 and 43.5% at 24 and 72 hours, respectively, after administration of thiamine ($P > 0.05$). Whole kidney weight as percentage of body weight at 2 and 3 weeks of thiamine deficiency was similar to that of pair-fed rats but rose by 12% at 4 weeks, 40% at 5 weeks and remained elevated by 27.3 and 33%, respectively, at 24 and 72 hours after thiamine administration ($P < 0.05$). Whole kidney and medullary weight as percentage of body weight was comparable in 5-week pair-fed and normally fed controls. Cortical and medullary protein concentrations in five sets of thiamine-deficient symptomatic and pair-fed control rats were similar ($P > 0.05$)

Table 2 depicts the in vitro effect of thiamine pyrophosphate on renal cortical and medullary pyruvate decarboxylase and transketolase activity in thiamine deficiency. In agreement with the in vivo effects of exogenous thiamine, addition of thiamine pyrophosphate to kidney tissue from symptomatic thiamine-deficient rats restored pyruvate decarboxylase activity of both cortex and medulla to levels seen in the pair-fed controls. By contrast, transketolase activity in renal cortex and medulla of the thiamine-deficient rats was not enhanced. Thiamine hydrochloride in vitro was without effect on pyruvate decarboxylase activity of thiamine deficient kidney.

The lack of a significant in vitro TPP effect for pyruvate decarboxylase activity in kidneys of the pair-fed controls attests to the absence of significant thiamine deficiency in the kidneys of these animals. This is further corroborated by inability to raise the kidney pyruvate decarboxylase activity in 4 sets of pair-fed controls given supplemented thiamine intraperitoneally during weeks 4 and 5 of the experimental period. The small decreases in renal pyruvate decarboxylase and cortical transketolase activity in pair-fed animals probably represent an effect of semistarvation on the respective apoenzymes.

TABLE 2

In vitro effect of thiamine pyrophosphate (TPP) on regional renal pyruvate decarboxylase and transketolase activity in thiamine deficiency [1]

	Cortex		Medulla	
	Pair-fed control	Thiamine deficient	Pair-fed control	Thiamine deficient
Pyruvate decarboxylase activity	189.6 ± 27.4	46.7 ± 6.01	70.0 ± 10.7	21.9 ± 1.81
TPP effect (increase)	3.1%	339.7%	6.1%	301.3%
Depression in thiamine deficient animals				
Before TPP		72.0%		63.8%
After TPP		0.6%		None
Transketolase activity	69.0 ± 6.13	7.27 ± 0.95	35.2 ± 2.61	2.80 ± 0.41
TPP effect (increase)	2.1%	4.7%	1.6%	None
Depression in thiamine deficient animals				
Before TPP		89.6%		92.0%
After TPP		89.2%		92.5%

[1] Tissue obtained from four sets of symptomatic rats kept for 4.5 to 5 weeks on a thiamine-deficient diet, and from their asymptomatic pair-fed controls. The data are given as the mean ± SE of four sets of kidneys each assayed in triplicate. The units for both enzymes are given in table 1. Twenty micromoles thiamine pyrophosphate per incubation flask was employed for the TPP effect.

TABLE 3

Effect of thiamine deficiency on regional renal metabolic rate [1]

	Lactate [2]	Δ Lactate	ATP [2]	Δ ATP	Δ ~ P
Cortex					
Normal control	1.97 ± 0.36	1.10 ± 0.11	1.57 ± 0.04	0.90 ± 0.09	2.90
Pair-fed control	2.05 ± 0.36	1.31 ± 0.25	1.73 ± 0.04	0.86 ± 0.09	3.03
Thiamine deficient	2.59 ± 0.34	1.18 ± 0.25	1.71 ± 0.07	0.81 ± 0.16	2.82
Medulla					
Normal control	2.56 ± 0.51	2.30 ± 0.22	1.72 ± 0.04	0.56 ± 0.10	3.42
Pair-fed control	2.60 ± 0.43	3.33 ± 0.96	1.66 ± 0.05	0.48 ± 0.07	4.29
Thiamine deficient	2.81 ± 0.40	2.90 ± 0.33	1.12 ± 0.06	0.34 ± 0.07	3.58

[1] Symptomatic rats after 4.5 to 5 weeks of thiamine deficiency and their pair-fed controls were used. Details of tissue procurement, assay, calculation and significance of Δlactate, ΔATP and Δ ~ P are given in the Experimental Procedure.

[2] Lactate and ATP values are expressed in μmoles/gram wet weight. The values represent the means ± SE of 5 to 7 separate lactate and 10 ATP determinations. Assay of blood lactate in nine sets of thiamine-deficient and pair-fed controls indicates that correction of kidney lactates for the trapped blood lactate would not significantly alter the comparison of the values given above, hence such a correction was not carried out.

Table 3 shows the renal cortical and medullary lactate concentrations, anaerobic glycolytic rate (Δlactate) and energy utilization (ΔATP) in symptomatic thiamine-deficient and control rats. The lactate concentrations in the thiamine deficient animals were slightly, but not significantly, increased, whereas Δlactate, ΔATP and overall metabolic rate (Δ ~ P) for either cortex or medulla were comparable for the three groups of animals ($P > 0.05$). The data further show that ATP decreases more rapidly in ischemic renal cortex than in the medulla, whereas anaerobic glycolysis is greater is ischemic medulla.

In five sets of rats kept for 4 weeks on a thiamine-deficient diet, the BUN, serum Na and K were (mean ± SE) 35.2 ± 7.47 mg/100 ml, 142.2 ± 2.16 and 4.50 ± 0.26 mEq/liter, respectively; in pair-fed controls the values were 22.3 ± 3.42 mg/100 ml, 139.2 ± 1.68 and 4.18 ± 0.26 mEq/liter. These sets of values do not differ significantly, although the BUN in the thiamine-deficient rats tends to be higher.

DISCUSSION

The present study clearly demonstrates that the renal medullary ATP concentration falls significantly and progressively with increasing thiamine deficiency (fig. 1). The validity of this finding is attested to by the following observations: 1) similar results are obtained if the data are expressed in terms of tissue wet weight or its protein concentration; 2) no morphologic abnormalities which could alter the reference basis for the ATP determination were noted in thiamine-deficient renal medulla by light microscopy [7] and 3) the ATP concentration rapidly reverted to normal after a single thiamine injection, indicating that the initial ATP depression was not due to a disproportionately greater general malnutrition of the thiamine-deficient rats. A nonspecific renal inhibitor of the ATP assay is ruled out by normal ATP levels in contiguous renal cortex and by the demonstration of full recovery of exogenous ATP added in vitro to ATP-depleted renal medulla of thiamine-deficient rats. Preliminary studies in our laboratory indicate that cardiac ATP concentration likewise falls significantly in severe thiamine deficiency and reverts to normal within 24 hours after a single injection of thiamine (5). Hepatic ATP in the symptomatic thiamine-deficient rats decreases slightly but significantly, whereas duodenal and ileal ATP are comparable to control values.[8]

The decreased ATP concentration in the renal medulla of thiamine-deficient animals could be the result of decreased synthesis, increased utilization of this nucleotide or a combination of these effects. The normal rate of anaerobic glycolysis and lack of increased ATP utilization in the medulla, as well as the cortex, of symptomatic thiamine-deficient rats (table 3), imply a normal metabolic rate and strongly argue against an increased utilization of high energy phosphates in the kidneys of these

[7] Unpublished observations.

[8] Manuscript in preparation.

animals. On the other hand, a number of findings obtained in this study are consistent with decreased synthesis of ATP via decreased pyruvate decarboxylation and subsequent impairment of the TCA cycle. First, during the development of decreased medullary ATP, medullary pyruvate decarboxylase activity decreased substantially. The observed degree of fall in the activity of this enzyme is quite comparable to that noted in whole kidney by Dreyfus and Hauser (7). While transketolase activity also declined markedly, the importance of this enzyme system for cellular synthesis of high energy phosphates is tenuous (23) and the hexose monophosphate shunt, wherein transketolase participates, is of only very minor significance in overall renal metabolism (8, 9). The possibility that a depression of α-ketoglutarate decarboxylase contributes to a decrease in energy synthesis has not been investigated. Second, the rapid return of medullary ATP to normal levels, after thiamine administration, correlates well with an impressive rise in medullary pyruvate decarboxylase activity (fig. 1). Contrariwise the transketolase activity rose only slightly at 24 hours and not at all at 72 hours after thiamine administration. This differential in-vivo effect of thiamine on these two enzymes is corroborated fully by our in vitro studies with both renal medulla and cortex (table 2). In addition, the studies with heart of thiamine-deficient rats likewise showed a correlation of ATP recovery after thiamine administration with a substantial rise in pyruvate decarboxylase, but not of transketolase, activity (5). These data therefore suggest that the depression of renal medullary ATP in thiamine deficiency is due primarily to decreased ATP synthesis, most likely as a result of decreased decarboxylation of pyruvate. This interpretation implies that CO_2 fixing reactions in thiamine-deficient medulla are insufficient to sustain normal function of the TCA cycle, a concept that requires confirmation by actual analysis of the TCA cycle intermediates in the medulla.

The present study also documents the variable regional renal response to thiamine deficiency. As shown in table 1, a decrease in renal cortical pyruvate decarboxylase and transketolase activity virtually identical to that found in the medulla failed to depress the renal cortical ATP. Perhaps the most reasonable explanation for this "resistance" of the cortex to the effects of thiamine deficiency is its high dependence on metabolism of fatty acids (9). This would enable the cortex to bypass the "block" in pyruvate conversion to acetyl CoA resulting from thiamine deficiency and replenish the TCA cycle from the utilization of fat. By contrast the medulla metabolizes primarily carbohydrates and is thus more vulnerable to impairment of their utilization (9). This concept is consistent with the proposed causative correlation between severe impairment of pyruvate decarboxylation and a depression of the ATP concentration in the renal medulla and the heart (5), and it may also explain by the same mechanism the protective effect of a high fat (12, 13) and moderate alcohol intake (24, 25) on low-thiamine encephalopathy.[9]

Our data on regional renal metabolism are in general agreement with the in vitro studies of Lee et al. (9) and the in vivo data of Needleman et al. (20). As shown in table 3, the renal medulla elaborated significantly more lactate and sustained a lesser ATP fall in the postischemic state than the cortex. This is consistent with the well-known higher rate of anaerobic glycolysis in the medulla which presumably assures the generation of ATP even during anoxia. It is of interest that the same rate of anerobic glycolysis (Δlactate) was maintained in the thiamine-deficient and in control rats. This suggests that the Embden-Meyerhof pathway, which is the principal glycolytic route in kidney, is not significantly impaired in thiamine deficiency. The lack of a definite rise in renal lactate despite a major depression of pyruvate decarboxylase activity in thiamine-deficient kidney is unexpected and contrary to results obtained by us previously in brain (2). It may be due to conversion of lactate to other metabolites or its diffusion out of kidney.

[9] In the brain, however, circumvention of decreased pyruvate decarboxylase activity by metabolites of fat and alcohol would protect by mechanisms other than an effect on brain ATP concentration, which remains normal in thiamine-deficient rats with neurologic signs (2).

Finally, two comments are pertinent to this discussion. First, the kidney as a whole, as well as the renal medulla, showed hypertrophy with severe thiamine deficiency and this could not be accounted for by increased tissue water content. The stimulus and mechanism for this hypertrophy are unknown, although hypokalemia can be ruled out by the data obtained. Second, the functional significance, if any, of the changes in medullary ATP or of the other enzyme alterations noted in thiamine-deficient kidney is unknown. It is important to emphasize that, because of the renal hypertrophy at 5 weeks of thiamine deficiency, the total medullary ATP content, as percentage of body weight, at that stage is normal. Prior to that time renal hypertrophy does not compensate for the depressed medullary ATP concentration. Future studies are required to determine if the kidney in thiamine deficiency alone, or in combination with other renal disorders, exhibits a significant functional impairment which can be correlated with these biochemical alterations.

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Effect of Dietary Molybdenum and Sulfate upon Copper Metabolism in Sheep [1,2]

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ABSTRACT The effects of 0.4% dietary inorganic sulfate, or a combination of 50 ppm molybdenum plus 0.4% sulfate, upon plasma clearance of intravenously injected ^{64}Cu, and its uptake by the liver and incorporation into ceruloplasmin, were studied in Florida native sheep. When both sulfate and molybdenum were present in the diet, a reduced uptake of radiocopper by the liver and an impairment in its utilization for ceruloplasmin synthesis occurred. This resulted in a slower removal of radiocopper from the plasma. Stable copper in the liver and in the ceruloplasmin fraction of plasma was significantly reduced in the sheep receiving both sulfate and molybdenum. All data indicated a metabolic interference with copper by sulfate and molybdenum in the liver. As a tentative explanation of the mechanism of this interference, either an impairment of copper uptake by liver cells, or a primary intracellular metabolic disturbance in the synthesis of copper-protein compounds including ceruloplasmin, or both, are postulated. Dietary inorganic sulfate, in the absence of supplemental molybdenum had no effect upon the parameters of copper metabolism studied.

It has been well established that dietary molybdenum and sulfate affect the storage of body copper under both field and experimental conditions (1). The sites where, and the mechanisms by which, molybdenum and sulfate influence copper metabolism, however, are largely unknown (2). In the present study, ^{64}Cu of high specific activity bound to plasma protein in vitro was injected intravenously into sheep receiving various dietary molybdenum and sulfate treatments, and the pattern of its clearance from the plasma and its uptake by the liver and other tissues were determined. The utilization of the injected ^{64}Cu for ceruloplasmin synthesis was also determined.

MATERIALS AND METHODS

Due to the short half-life of ^{64}Cu (12.9 hours) and the time required to handle animals and samples, several separate trials were conducted to obtain the desired data. Similar results were found for different trials and the data were pooled and evaluated statistically by analysis of variance. Sixty Florida native wethers, averaging 32 kg in body weight initially, were used; animals in each trial were of similar age and body weight. All animals were examined for internal parasites prior to and during the experiments, and were dosed with thiabendazole when necessary. The sheep were housed in individual pens during a preliminary feeding period of 4 months, and in metabolism stalls during the radioactive tracer studies. Three dietary treatments were studied, including: a) the basal or control diet (table 1) [6] containing 12 to 14 ppm of copper and 0.03% inorganic sulfate, both determined by analysis; b) basal diet plus 0.4% sulfate as sodium sulfate; and c) basal diet plus 0.4% sulfate as sodium sulfate and 50 ppm molybdenum as sodium molybdate. Eight hundred grams of feed were offered daily in two equal feedings to each animal and feed consumption was recorded. Tap water was provided ad libitum.

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[6] Appreciation is expressed to Dawe's Laboratories, Inc., Chicago, Illinois, for supplying vitamins A and D, and to Monsanto Chemical Company, St. Louis, Missouri, for supplying Santoquin.

TABLE 1

Composition of basal diet

Ingredient	
	%
Corn cobs and shucks, ground	26
Soybean protein [1]	10
Corn dextrose	20
Cellulose [2]	10
Corn starch	27
Corn oil [3]	4
Minerals [4]	3
Vitamins [5]	+
Variables [6]	+
	100

[1] Assay Protein C-1, Skidmore Enterprises, Cincinnati, Ohio.
[2] "Solka-floc," The Dicalite Company, New Orleans, Louisiana.
[3] Santoquin added at 0.0125% of total diet.
[4] Macrominerals added to the diet, expressed as percent were as follows: Ca, 0.40 ($CaHPO_4 \cdot 2H_2O$); P, 0.32 ($CaHPO_4 \cdot 2H_2O$); K, 0.35 (KCl); Na, 0.15 (NaCl); Mg, 0.050 (MgO). Trace minerals added expressed in ppm were as follows: Fe, 80 ($FeSO_4$); Zn, 50 ($ZnCO_3$); Mn, 25 ($MnCO_3$); Cu, 10 ($CuSO_4$); Co, 2 ($CoCO_3$); I, 5 (KI).
[5] Vitamins added per kg of diet: DL-alpha-tocopherol, 11 mg; vitamin A palmitate, 4400 IU; and vitamin D_2, 1100 IU.
[6] Na_2SO_4 and $Na_2MoO_4 \cdot 2H_2O$ were added to supply desired levels of sulfate and molybdenum. Starch was varied to adjust to 100%.

Radiocopper labeled plasma samples were prepared by adding 5 mCi of ^{64}Cu (cupric nitrate, specific activity 25 to 35 mCi per mg) to 5 ml of sheep's plasma, mixing and incubating at room temperature for 30 minutes. To determine the activity administered, appropriate standards were prepared. After incubation, the ^{64}Cu-labeled plasma samples were injected intravenously and, beginning 3 to 5 minutes after injection, serial blood samples of 5 ml each were withdrawn with heparinized syringes from each sheep during a 30-hour period. The blood was centrifuged, and 2 ml of plasma were pipetted into plastic tubes for counting in a well-type scintillation counter. To compare the ^{64}Cu curves obtained in the different animals, the data were expressed as a percentage of the calculated administered radioactivity at time zero.

The rate of incorporation of injected ^{64}Cu into ceruloplasmin was determined by the following procedure. Twenty-four hours after the intravenous administration of plasma which had been labeled in vitro with 10 mCi of ^{64}Cu, 150 to 200 ml of blood were withdrawn in heparinized bottles from sheep receiving either the control or molybdenum-sulfate diet. The blood was centrifuged and the plasma obtained. Eighty to 100 ml of plasma were immediately injected intravenously into normal sheep which were free of radioactivity and were consuming the control diet. Beginning approximately 2 minutes after administration, serial blood samples of 5 ml each were withdrawn at intervals for 25 hours. The samples were centrifuged and the plasma radioassayed. The percentage of administered ^{64}Cu dose in the ceruloplasmin fraction was calculated by extrapolating back the second component of the clearance curves when plotted on semilog paper. This procedure was based on the fact that the clearance of ceruloplasmin from the plasma has a half-life of several days, while the inorganic copper or the copper loosely bound to albumin has a plasma half-clearance time of only minutes (3–4).

The uptake of ^{64}Cu by the liver was determined by slaughtering sheep at various time intervals after injection of the tracer. Whole organs were radioassayed in a 4-"Pi" liquid scintillation detector. Appropriate standards with similar geometry and volume of the samples were used to calculate the percentage of injected activity present in the samples. In all radioassays where necessary, corrections for physical decay of ^{64}Cu were made.

Stable copper content of the liver and plasma samples was measured by atomic absorption spectrophotometry (5). The liver samples were ashed and the resulting ash dissolved in a dilute HCl solution. Total plasma copper was determined directly after diluting 1:1 with triple-distilled water. Direct reacting copper, or inorganic copper of the plasma was determined by the method of Gubler et al. (6). Ceruloplasmin copper was assumed to represent the difference between total and direct reacting copper. The experimental data were tested by analysis of variance with significant differences between means determined by the method of Duncan (7).

RESULTS

The plasma clearance patterns of ^{64}Cu as influenced by treatment are shown in figure 1. When the experimental data were examined by computer, it was verified that the plasma clearance of ^{64}Cu for all

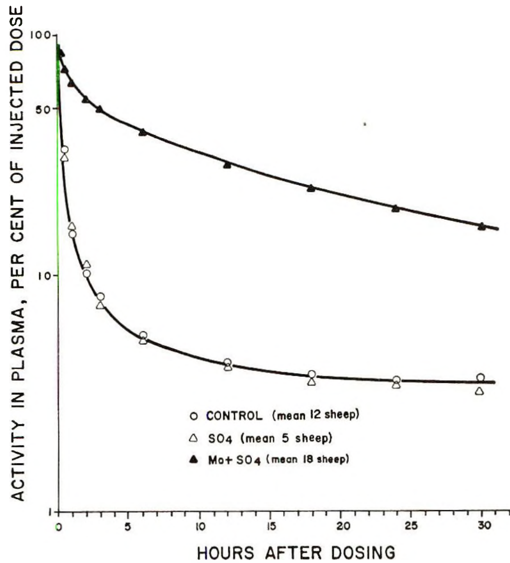

Fig. 1 Plasma clearance of intravenously injected ^{64}Cu as influenced by dietary molybdenum plus sulfate. Experimental diets fed for 120 days.

treatments could be characterized by a 3-term exponential function similar to: $A_1e^{-k_1t} + A_2e^{-k_2t} + A_3e^{-k_3t}$. A distinct difference, however, was found among the terms of the sheep fed molybdenum plus sulfate when compared with those for the other two treatment groups. The data for the compartmental parameters and rate constants of ^{64}Cu plasma clearance for the sheep fed the control diet and for those fed added dietary molybdenum plus sulfate are summarized in table 2. A multi-term exponential function of ^{64}Cu plasma clearance suggests that there is a feedback of the tracer from the compartments into which it goes, mainly in the animals fed molybdenum plus sulfate. This fact does not permit the plasma inorganic copper transport rate to the tissues to be calculated with accuracy. If, however, a tentative value is obtained using the data in table 2 and the inorganic plasma copper levels for the same group of animal (table 5), the amount of inorganic copper removed per unit time from the plasma to the tissues was more than two times higher in the control animals than in those fed the diet containing molybdenum plus sulfate.

The data are plotted in figure 2 for plasma clearance of ^{64}Cu plasma which was labeled in vivo by either control or molybdenum-sulfate fed donor animals and then transfused to normal recipient animals. The plasma was withdrawn from the donor animals 24 hours after injecting the tracer. By extrapolating back the second component of the clearance curves, the figure shows that at the 24-hour period about 66% of the activity remaining in the plasma of the control donor animals was due to ^{64}Cu already synthesized into ceruloplasmin. However, only 16.5% of the activity remaining in the plasma of the donor animals fed molybdenum plus sulfate was in the ceruloplasmin fraction. This difference was significant ($P < 0.01$).

The values for liver uptake of ^{64}Cu 24 hours after injection of the tracer are summarized in table 3. About 80% of the injected dose was taken up by the liver for both the basal and the basal-plus-sulfate diet-fed groups while in the molybdenum-sulfate fed group only 33.1% of the activity administered was found in the liver. The stable liver copper content (table 4) was similar for sheep fed both the basal and the basal plus sulfate diet and was higher ($P < 0.01$) than that found for sheep fed the basal diet plus molybdenum and sulfate.

TABLE 2

Compartmental clearance and rate constants obtained from plasma clearance curves of intravenously injected ^{64}Cu (fig. 1) for sheep fed basal or basal plus molybdenum and sulfate diets

Dietary treatment		A_1	A_2	A_3	k_1	k_2	k_3
			% of dose			*hr^{-1}*	
Basal	Mean	90.5	5.5	4.0	2.77	0.20	0.0034
	SD [1]	6.2	0.8	0.5	0.29	0.03	0.0006
Basal + SO_4 + Mo	Mean	29.3	41.3	29.4	0.71	0.12	0.0190
	SD [1]	5.2	7.4	5.7	0.09	0.02	0.0033

[1] Standard deviation.

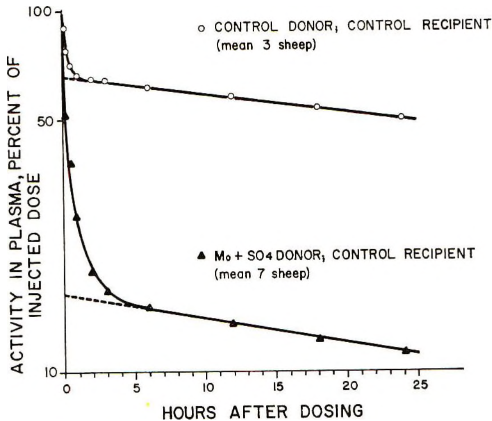

Fig. 2 Plasma clearance by normal sheep of intravenously injected ^{64}Cu-labeled plasma in vivo. The plasma samples were obtained from control and molybdenum-sulfate fed animals 24 hours after injecting the tracer and immediately transfused to control animals free of radioactivity.

Plasma copper levels obtained 120 days after starting the animals on experimental diets are summarized in table 5. Total plasma copper values were not significantly different and treatment means ranged between 101.9 and 112.3 μg per 100 ml of plasma. The group fed molybdenum plus sulfate, however, had a lower ($P < 0.01$) ceruloplasmin copper level and a higher ($P < 0.01$) level of plasma inorganic copper than did the basal or basal plus sulfate groups.

Feed intake and body weight gains were not different due to dietary treatment in any of the trials. For the entire feeding period of 120 days, average feed consumption was about 700 g per animal daily and total weight gains were 8.6, 9.2 and 9.0 kg for the sheep fed the basal, basal plus sulfate, and basal plus sulfate and molybdenum diets, respectively.

DISCUSSION

Copper, in the inorganic form, is transported through the blood mainly in loose combination with plasma albumin, and to a much lesser degree, bound to amino acids (3). This form of copper normally represents only 5 to 20% of total plasma copper and is readily taken up by the liver. The degree to which liver uptake occurs varies with species, but in general it is estimated

TABLE 3

Liver uptake by sheep of ^{64}Cu 24 hours after intravenous injection as influenced by dietary molybdenum and sulfate [1]

Item	Dietary treatment		
	Basal	Basal + SO_4	Basal + SO_4 + Mo
No. of animals	5	5	18
Mean [2]	79.6 [a]	82.1 [a]	33.1 [b]
Standard deviation	8.0	5.3	8.1

[1] Data expressed as percentage of injected activity. Experimental diets fed 120 days.
[2] Means with different superscripts are significantly different ($P < 0.01$).

TABLE 4

Stable liver copper of sheep as influenced by dietary molybdenum and sulfate [1]

Item	Dietary treatment		
	Basal	Basal + SO_4	Basal + SO_4 + Mo
No. of animals	9 [2]	5	15
Liver copper [3]			
Concentration (dry wt), ppm	1,151 ± 359 [a]	1,157 ± 254 [a]	301 ± 111 [b]
Whole organ, mg	158.3 ± 43.5 [a]	181.5 ± 54.0 [a]	47.3 ± 20.4 [b]

[1] Experimental diets fed 120 days.
[2] Five animals included in table 2 plus 4 with similar treatment.
[3] Mean values plus standard deviations with different superscripts are significantly different ($P < 0.01$).

TABLE 5

Average inorganic, ceruloplasmin and total plasma copper values for sheep as influenced by dietary molybdenum and sulfate [1]

Item	Dietary treatment		
	Basal	Basal + SO_4	Basal + SO_4 + Mo
No. of animals	8	5	10
Plasma copper [2]			
Inorganic	24.9 ± 7.4 [a]	14.6 ± 5.1 [a]	57.4 ± 5.8 [b]
Ceruloplasmin	87.4 ± 7.1 [a]	94.8 ± 7.9 [a]	44.5 ± 6.3 [b]
Total	112.3 ± 10.2 [a]	109.4 ± 9.6 [a]	101.9 ± 10.2 [a]

[1] Experimental diets fed 120 days.
[2] Data expressed as μg Cu/100 ml plasma. Means plus standard deviations with different superscripts are significantly different ($P < 0.01$).

to be between 70 and 90% in 24 hours (3, 8). This was confirmed by the present study since about 80% of the plasma ^{64}Cu was accumulated in the liver during the first 24 hours after injection in control animals.

Dick (9) estimated that in sheep about 75% of total body copper is in the liver. Aside from its copper storage function, the liver is also the site of ceruloplasmin formation (10). It has been stated, and experimentally supported, that ceruloplasmin could be the most important, if not the only, source of copper available to the tissues (3, 8, 10). The results from the present study indicate that dietary molybdenum plus sulfate affected at least two critical steps of copper metabolism, including the liver uptake of copper and copper utilization for ceruloplasmin synthesis. This is suggested by a slower removal of intravenously injected ^{64}Cu from the plasma and a reduced use of the copper for ceruloplasmin synthesis. This latter observation is in agreement with the lower values of stable copper in the ceruloplasmin fraction of plasma copper in the same animals. The reduced stable copper content of the liver from animals fed molybdenum plus sulfate is also consistent with the lower hepatic uptake of the injected radiocopper in those animals. The fecal excretion of the injected tracer (fig. 3), indicated that animals fed molybdenum plus sulfate excreted somewhat less copper initially, presumably via the bile, than did the control animals. The shape of the excretion curves, however, suggests that in a longer observational period a greater amount of radiocopper would have been excreted by animals fed molybdenum plus sulfate. When excretion was calculated on the basis of the amount of tracer present in the liver, the molybdenum-sulfate animals excreted about three times more radiocopper in the feces than did the control animals. This finding was tested further by determining in another assay, the activity present in the liver, gall bladder, and sections of duodenum, jejunum and ileum plus contents, from control and molybdenum-sulfate fed animals as 2, 12, 24, and 48 hours after injecting radiocopper. On the basis of the amount of radiocopper in the liver, the activity present in the gall bladder and segments of the small intestine was between 5 and 10 times greater in the molybdenum-

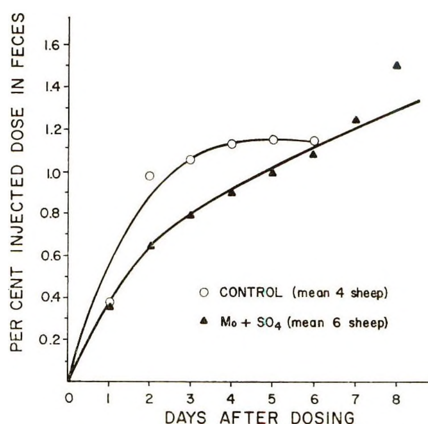

Fig. 3 Daily fecal excretion of ^{64}Cu after a single intravenous injection to sheep as influenced by feeding a control or a molybdenum-sulfate diet for 120 days.

sulfate fed animals than in the control animals (table 6). This suggests that biliary excretion of copper was increased by molybdenum plus sulfate. If biliary copper is poorly reabsorbed in sheep as it is in the rat (11), fecal losses of copper in ruminants fed molybdenum plus sulfate could be of importance.

While the results from the present experiments do not offer conclusive evidence concerning the mechanisms by which molybdenum plus sulfate affect the uptake of copper by the liver, two postulates are advanced. Either copper cannot enter the liver cells and consequently its storage and utilization for ceruloplasmin synthesis are indirectly affected; or there is a primary intracellular metabolic defect preventing the synthesis of storage-copper-complexes and ceruloplasmin. Both mechanisms may occur and, in either case, the mineral should be partially returned to the plasma and excreted by way of the bile. On the basis of recent research with both ruminants and nonruminants, Dowdy and Matrone have hypothesized that molybdenum complexes with copper and that the copper bound in this form is biologically inactive (12, 13).

According to a recently proposed model for copper metabolism in the rat (14), copper is handled by the liver through three different and partially independent physiological compartments. One compartment leads to its excretion via the bile, another to temporary storage and the third one, which has a limited capacity, to incorporation into ceruloplasmin. Under these assumptions a lower uptake of copper by the storage and ceruloplasmin compartments or a metabolic disturbance of copper within these compartments should increase biliary excretion. It has been shown in rabbits, however, that high levels of dietary molybdenum plus sulfate resulted in a decreased liver uptake of copper, an inhibition of ceruloplasmin synthesis, and an extremely low biliary excretion of copper (15). These conflicting results between species on the biliary excretion of copper are, however, consistent with the fact that in rabbits a high intake of molybdenum plus sulfate leads to the accumulation of copper in the tissues, mainly in the brain (15, 16), while in ruminants body tissues become depleted of copper.

If tissues depend on copper supplied in the form of ceruloplasmin, this could ex-

TABLE 6

Radioactivity present in the gall bladder and sections of duodenum, jejunum and ileum after intravenous injection of ^{64}Cu as influenced by dietary molybdenum plus sulfate [1]

Hours after dosing	Dietary treatment	Tissue							
		Gall bladder [2]		Duodenum [3]		Jejunum [3]		Ileum [3]	
		A [4]	B [5]	A [4]	B [5]	A [4]	B [5]	A [4]	B [5]
2	Basal	0.03	0.08	0.57	0.99	0.69	1.72	0.15	0.34
	Mo + SO_4	0.03	0.27	0.58	1.47	0.50	4.51	0.16	1.52
12	Basal	0.02	0.03	0.24	0.46	0.14	0.24	0.20	0.34
	Mo + SO_4	0.02	0.06	0.48	0.80	0.54	1.40	0.46	1.13
24	Basal	0.03	0.04	0.39	0.59	0.31	0.44	0.27	0.41
	Mo + SO_4	0.07	0.15	0.69	1.50	0.68	1.52	0.40	0.94
48	Basal	0.01	0.02	0.49	0.67	0.23	0.38	0.16	0.27
	Mo + SO_4	0.08	0.24	0.78	1.80	0.39	0.87	0.34	0.62

[1] Experimental diets fed 120 days. Each value represents one animal for each time interval and each treatment.
[2] Gall bladder plus contents.
[3] Data represent 100 g of fresh tissue and contents.
[4] Data expressed in percent of injected activity.
[5] Data expressed in percent of total activity in liver.

plain why copper-deficiency symptoms are observed in animals which are fed molybdenum plus sulfate but which may have a considerable amount of stored tissue copper and even increased inorganic copper levels in plasma, as reported by Dick (17). The fact that the half-time of ceruloplasmin in plasma is only a few days (3) suggests that a continuous supply for tissues is needed, making it possible to produce a "copper deficiency" at the tissue level in a short time if ceruloplasmin synthesis is affected.

The parameters of copper metabolism, studied in the present work, strongly indicate that they were not affected by inorganic sulfate when supplemental molybdenum was absent from the diet, confirming earlier observations by Dick (17).

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Effect of Cold Exposure on the Response of Rats to a Dietary Amino Acid Imbalance [1,2]

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ABSTRACT Relationships among food intake, growth, liver serine-threonine dehydratase activity (S-TDH) and plasma amino acid concentrations were studied at intervals in cold-exposed rats fed a diet having an amino acid imbalance (5% casein plus 6% amino acid mixture minus histidine). Within 4 hours after rats had eaten a meal of this diet their plasma histidine concentration fell and concentrations of other indispensable amino acids and serine rose; subsequently, food intake and growth were depressed for 2 days. The S-TDH was low initially, rose to a maximum, then fell; but after 9 days was still seven-fold greater than at day zero. As time progressed food intake and growth increased, but plasma histidine concentration remained low while concentrations of other indispensable amino acids remained elevated. The elevated metabolic activity of cold-exposed rats apparently permits them to consume a large quantity of imbalanced diet before their plasma amino acid pattern becomes sufficiently abnormal to elicit a signal leading to curtailment of food intake.

Food intake and growth depressions associated with a marked decrease in the concentration of the limiting amino acid, and elevated concentrations of the indispensable amino acids in blood plasma are early effects of ingestion of a diet with an amino acid imbalance (1–3). Rats exposed to a cold environment, however, readily consume diets with moderate imbalances and grow at rates similar to rats fed appropriate, balanced control diets (4–6). They also degrade amino acids more rapidly than rats kept in a warm environment (5–7), and the plasma amino acid pattern of cold-exposed rats fed a diet in which an imbalance was produced by only 1% of amino acids was similar to that of controls fed a balanced basal diet (6). These observations suggested (5) that cold-exposed animals catabolized the imbalancing portion of the amino acid mixture preferentially and used it for heat production while the remaining balanced portion was used efficiently for protein synthesis. Nevertheless, rats kept in a cold environment and offered a choice between a protein-free diet and a diet with an imbalance (8) showed a distinct preference for the protein-free diet, just as rats offered the same choice in a warm environment (9, 10). Further, cold exposure did not prevent a transitory depression in the food intake of rats fed a diet with a severe imbalance (8) or a diet containing an excessive amount of leucine (5, 11).

Elevated food intake, to satisfy the increased energy need in a cold environment, would result in an increased intake of the limiting amino acid and, hence, in consumption of a greater quantity of amino acids that could be utilized for tissue protein synthesis. This, together with the increased ability of the cold-exposed rat to degrade amino acids (5–7), should permit the cold-exposed rat to consume more of a diet with an amino acid imbalance or tolerate a greater dietary imbalance before the concentrations of amino acids in body fluids were altered enough to result in food-intake depression. However, the selection of a protein-free diet over a diet with an imbalance by cold-exposed rats (8) would indicate that: 1) the plasma amino acid pattern of cold-exposed rats fed the diet with this amino acid imbalance was abnormal even though their food intake was not depressed; 2) whatever enhancement of amino acid oxidation occurred in cold-

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exposed rats, it was not enough to restore the plasma amino acid pattern of rats fed the diet with this imbalance (8) to normal; and 3) that the appetite-stimulating effect of the high energy need of cold-exposed rats can override the appetite-depressing effect of an amino acid imbalance.

To investigate these possibilities, alterations in food intake, growth, liver serine-threonine dehydratase activity and plasma amino acid concentrations were determined at intervals in rats exposed to a cold environment and fed a low protein diet containing an amino acid mixture devoid of histidine to create an imbalance. Serine-threonine dehydratase activity was chosen as representative of enzymes that increase in activity under conditions in which amino acid degradative ability is enhanced (12–18).

EXPERIMENTAL

Male rats of the Holtzman strain, weighing approximately 100 g, were individually housed in screen-bottom cages at 9° in a room with a 6-hour dark, 18-hour light cycle. All rats were weighed and fed each day just before the beginning of the dark hours. During the first 13 days of cold exposure, rats were fed the basal, low protein diet and water, ad libitum. At the end of this time the rats weighed an average of 120 g. On day 14 the rats were divided into two groups and were fed either the basal or the imbalanced diet for 9 days. Food intake and body weight were recorded daily for each rat.

The basal diet contained: (in percent by weight) casein, 5; L-methionine, 0.3; L-threonine, 0.2; mineral mixture, 5; corn oil, 5; vitamin mixture, 0.5; choline chloride, 0.2; and a cornstarch-glucose monohydrate mixture in the ratio 1:1 to make up 100%. The mineral and vitamin mixtures have been described previously (19). A histidine imbalance was created by adding to the basal diet 6%, by weight, of a mixture of amino acids devoid of histidine. The amino acid mixture contained: (in percent) L-methionine, 5; L-phenylalanine, 15; L-leucine, 15; L-isoleucine, 10; L-valine, 10; L-lysine·HCl, 15; L-arginine·HCl, 10; L-threonine, 7.5; L-tryptophan, 2.5; and Na acetate, 10. When the amino acid mixture was included in the basal diet, adjustments were made in the carbohydrate content. The cornstarch-glucose monohydrate mixture was substituted for casein in the protein-free diet. All diets were fed in the agar-gel form (19).

For enzyme and plasma studies, an amount of the basal or the imbalanced agar-gel diet equivalent to 5 g of dry matter was placed in the cage of each rat just before the beginning of the dark period. Two hours later food intake was checked, and from the rats that had consumed all the diet within this time, five receiving each diet were selected. Blood and liver were taken after another 2 hours, i.e., 4 hours after initiation of feeding. The remaining rats were then fed their respective diets ad libitum for the remainder of the day. This procedure was followed on the day the rats were divided into groups and after they had ingested the diets ad libitum for 1, 2, 4 and 9 days. On day 13 of cold-exposure, before they were fed the experimental diets, all rats were given 5 g of the basal diet and 10 rats that ate the full amount in 2 hours were taken as zero-day controls. Rats fed the imbalanced diet for 1 and 2 days did not eat all of the 5 g in 2 hours. Therefore, rats consuming the largest amount of the imbalanced diet in 4 hours were selected; for rats fed the imbalanced diet for 1 day this amount averaged 2.5 g, and for those fed for 2 days, 2.7 g.

Rats were lightly anesthetized with ether; blood was obtained by heart puncture immediately thereafter and the liver was excised, weighed and chilled in 0.14 M KCl. Plasma amino acid concentrations were determined on pooled samples from each group using an amino acid analyzer[4] (20). Liver serine-threonine dehydratase activity was determined by a modification (20) of the procedure of Freedland and Avery (14) using serine as the substrate.

RESULTS

Both food intake and weight gain of the groups fed the basal or the imbalanced diet in the cold (fig. 1) were higher than for comparable animals fed these diets in a warm environment (21); nevertheless, on days 1 and 2, values for rats fed the im-

[4] Technicon Auto-analyzer, Technicon Corporation, Ardsley, N. Y.

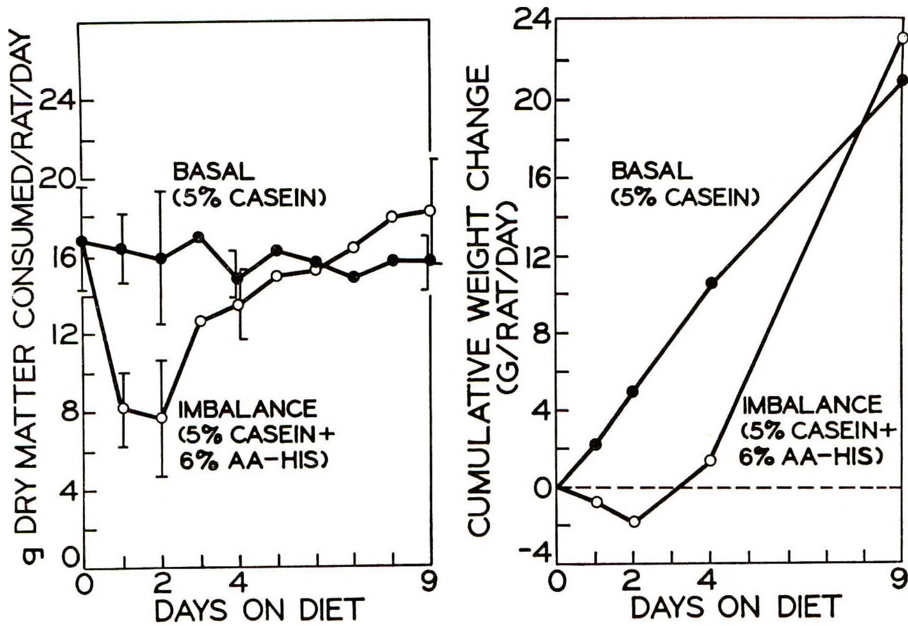

Fig. 1 Effect on food intake and growth of feeding a basal or an imbalanced diet to rats at 9° (five rats per group). Vertical lines indicate SEM.

balanced diet were lower than those for rats fed the basal diet. Food intake of rats fed the imbalanced diet in the cold was 9 g on day 1, about 49% of the zero-day value; but at room temperature (21) the value for this group was just over 3 g, 23% of the zero-day value. By days 3 or 4 food consumption and weight gain increased and continued to increase throughout the remainder of the study. Toward the end of the experiment rats fed the imbalanced diet were consuming about 2 g more food per day and were gaining more rapidly than those fed the basal diet. Results similar to those presented in figure 1 were obtained in three experiments.

Alterations in liver serine-threonine dehydratase activity are shown in figure 2. Serine-threonine dehydratase activity remained unchanged throughout the study in the livers of rats fed the basal diet. Enzyme activity in the livers of rats fed the imbalanced diet, however, had increased significantly by day 2 ($P < 0.05$), and even more by day 4 ($P < 0.02$); the value on day 9 was lower but was still sevenfold greater than that on day zero ($P < 0.01$). Enzyme activity of rats fed the imbalanced diet was significantly greater than that

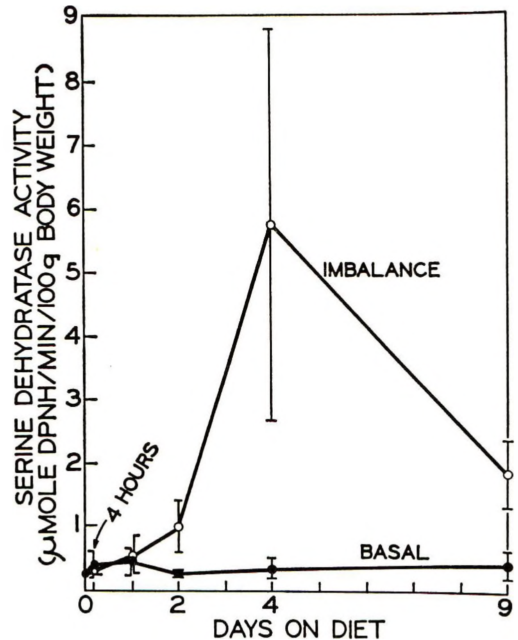

Fig. 2 Effect on serine dehydratase activity of feeding a basal or an imbalanced diet to rats at 9°. Each point represents the average of 5 rats except the value at day zero which is the average of 10 rats. Vertical lines indicate SEM.

of rats fed the basal diet on days 2, 4 and 9 ($P < 0.05$, $P < 0.02$, $P < 0.01$, respectively).

Alterations in the concentrations of several plasma amino acids are shown in figure 3. In rats fed the imbalanced diet the plasma concentration of histidine decreased markedly within 4 hours after their first meal; the same response was observed each day thereafter. Histidine concentration in the plasma of rats fed the basal diet varied from 9.9 to 11.6 μmoles/100 ml throughout the study. The concentration of eight indispensable amino acids (not including histidine and tryptophan) in the plasma of rats fed the imbalanced diet increased markedly within 4 hours after the initial meal, and was elevated at each interval thereafter. The total concentration of the eight indispensable amino acids in the plasma of rats fed the basal diet ranged from 228 to 260 μmoles/100 ml and on each day was considerably below that of rats fed the imbalanced diet. The ratio of the eight indispensable amino acids to histidine (I/H) was 90.7, 116.9, 237.4, 187.0 and 182.0 after the first meal and on days 1, 2, 4 and 9, respectively, for rats fed the imbalanced diet. This ratio ranged from 20 to 25 throughout the study for rats fed the basal diet. Changes in the concentration of total plasma amino acids were similar to changes in the concentration of the eight indispensable amino acids; values for rats fed the imbalanced diet were higher than those for rats fed the basal diet at each interval, the greatest difference being observed on day 2.

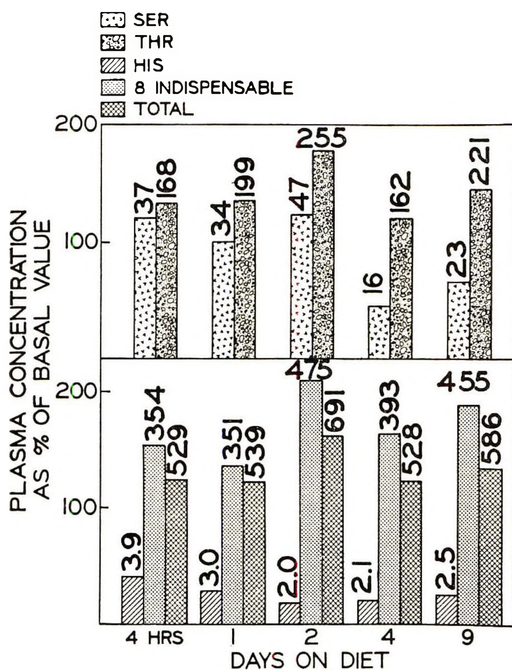

Fig. 3 Concentrations of histidine, eight indispensable and total amino acids (lower half), threonine and serine (upper half), in plasma of rats fed the imbalanced diet at 9°. Each bar represents the concentration of an amino acid or group of amino acids plotted as a percentage of the concentration of the same amino acid or group of amino acids in the plasma of rats fed the basal diet. Figures above each bar represent the concentration of the amino acids in micromoles per 100 ml of pooled plasma from five rats fed the imbalanced diet. Indispensable amino acids do not include histidine and tryptophan.

Threonine concentration in the plasma of rats fed the imbalanced diet was higher than that of rats fed the basal diet on each day of the study, and rose to the greatest value on day 2 when food intake was only 2.7 g. It did not rise to as high a value on days 4 and 9 when food intake was 5 g (fig. 3). Plasma serine concentration of rats fed the imbalanced diet on days 4 and 9 was less than that of rats fed the basal diet.

In another experiment six rats that had consumed the imbalanced diet and six that had consumed the basal diet for 16 days in a cold environment were offered a choice between a protein-free diet and their respective diets at the beginning of the dark period; the amounts of each diet consumed were measured 2, 4, 6 and 7 hours later. On the day prior to the food preference study the rats fed the imbalanced diet consumed 23 g of food while rats fed the basal diet consumed 22 g. The plasma amino acid patterns of two other groups treated in this way resembled those shown in figure 3 for rats fed the imbalanced and basal diets for 9 days. Rats previously fed the imbalanced diet preferred the protein-free diet (fig. 4); by 7 hours they had consumed only one-half as much of the imbalanced diet as of the protein-free diet. Rats previously fed the basal diet preferred the basal diet, but about one-third of their total intake was composed of the protein-free diet.

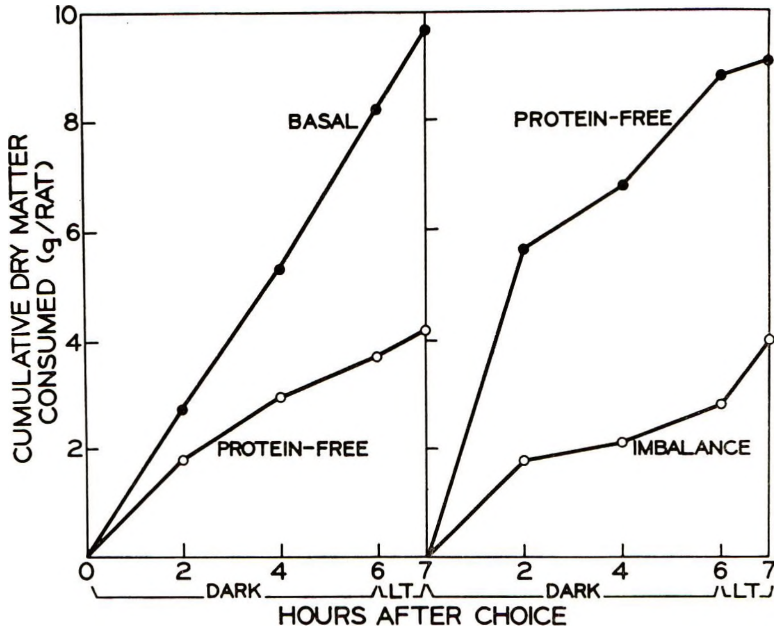

Fig. 4 Cumulative intake of protein-free diet and basal or imbalanced diet by cold-exposed rats allowed to choose between two diets.

DISCUSSION

The observation that the food intake of rats fed the imbalanced diet in a cold environment was less than that of controls fed the basal diet during the first few days differs from earlier observations (8) that the food intake of cold-exposed rats fed this imbalanced diet was not depressed, although rats fed a more severely imbalanced diet in the cold did eat less for a few days than controls fed the basal diet. Differences in pretreatment of the animals in the two experiments probably account for the differences in the results. In the earlier investigation the rats were fed laboratory chow for 1 week before they were fed the experimental diets, a procedure which increased their ability to adapt to an imbalanced diet (22); also, the rats were not adapted to the cold prior to receiving the imbalanced diet and during the first few days of the experiment both the control and experimental groups lost weight. Although no depression in the food intake of rats fed an imbalanced diet in a cold environment was observed by Klain et al. (5), Klain and Winders (6) and Beaton (4), they used diets that were less severely imbalanced (23) than those used in the experiments reported here.

Adaptation of rats to the imbalanced diet was more rapid in the cold environment than at room temperature. Elevation of food intake due to cold exposure would result in an increased intake of the limiting amino acid. This, together with the greater activity of amino acid-degrading enzymes due to cold exposure (7, 24) and to ingestion of the imbalanced diet (5, 6), could account for the more rapid adaptation.

When rats were fed the imbalanced diet in the present investigation, the initial low food intake was associated with low amino acid degradative ability, a fall in plasma histidine concentration and a rise in plasma indispensable amino acids; however, when food intake and amino acid degrading ability had increased subsequently, the plasma amino acid pattern still was abnormal. An explanation for the abnormal pattern of plasma amino acids both when food intake is depressed and when it subsequently increases has been suggested (21). Initially the ability of the rat fed the low protein, imbalanced diet to degrade amino

acids is low, and if an abnormal plasma amino acid pattern directly or indirectly triggers food intake depression (25), consumption of a relatively small amount of the imbalanced diet at this time would result in an abnormal plasma amino pattern and depressed food intake. As the ability to utilize amino acids increased, however, the amount of imbalanced diet necessary to produce a plasma pattern sufficiently abnormal to cause animals to stop eating would also increase. The responses observed in cold-exposed rats fit this pattern, suggesting that the factors influencing food intake of rats fed the imbalanced diet at room temperature also influence their food intake in a cold environment, but that the appetite-stimulating effect of the high energy need of cold-exposed rats overrides other stimuli making them less sensitive to any appetite-depressing effect of a dietary imbalance of amino acids.

Preference for the protein-free diet over the imbalanced diet, despite the fact that the animals were consuming and efficiently utilizing a large quantity of the imbalanced diet, was also observed previously (8), suggesting that whatever causes the altered food selection in the warm environment is apparently still operating in the cold environment. Although plasma amino acid concentrations of rats offered the dietary choice were not determined in the present investigation, an abnormal plasma amino acid pattern was observed in similarly treated animals, suggesting that the abnormal plasma pattern is responsible for the observed food selection. If so, it would appear that any increase in the rate of oxidation of amino acids by cold-exposed rats fed a diet with an amino acid imbalance is insufficient to prevent the plasma amino acid pattern from becoming abnormal, even though it may be sufficient to stimulate food intake, and that both food selection and plasma amino acid measurements provide more sensitive indices of amino acid imbalance than food intake and growth measurements in animals fed ad libitum.

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Influence of Dietary Carbohydrate on Duodenal Lesions in the Mouse [1,2]

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ABSTRACT A complete purified diet which promotes normal growth in rats and mice, and in which glucose is the only carbohydrate, increases the incidence and severity of avillous hyperplasia, a lesion of the duodenal mucosa occasionally encountered in stock-fed mice. The lesion is a focal thickening, composed of tubules derived from the crypts of Lieberkühn, and villi are absent. A further increase in incidence and severity of the lesion occurs in pantothenic acid deficiency, and chronic ulceration may then be superimposed upon it. Sucrose, substituted for glucose in the complete purified diet, causes a moderate decrease in incidence of the lesion. Cornstarch substituted for glucose causes a marked decrease in incidence. In neither case is severity significantly affected. In the pantothenate-deficient diet, cornstarch substituted for glucose reduces incidence of the lesion to approximately that produced by the glucose-containing complete diet. Severity, though markedly reduced by cornstarch in the pantothenate-deficient diet, still remains well above levels for the complete diet. The incidence of chronic ulcer in the starch-containing pantothenate-deficient diet fell from 30% to 18%, but this was not statistically significant.

Focal avillous hyperplasia of the crypts of Lieberkühn is the first of two lesions to be discussed in this report (1). It consists of grossly visible thickened plaques in the proximal duodenal mucosa. These lack villi, and microscopically are composed of large numbers of parallel tubules extending from the level of the muscularis mucosae to the surface. The second lesion is the deep chronic ulcer of pantothenic acid deficiency (2), believed to develop only in foci of avillous hyperplasia. Both these lesions are illustrated in figure 1. Avillous hyperplasia occurs to an inconspicuous extent in mice fed a natural foodstuffs stock diet. Its incidence and severity is far greater if a complete purified diet, containing 70.9% glucose, is fed in place of the stock diet; and a further marked increase occurs if the purified diet is deficient in pantothenic acid.

This aggravation of avillous hyperplasia by our complete purified diet, which long experience had demonstrated to be satisfactory in all other respects, provoked a search for an explanation; and, after certain other factors had been tested and eliminated, the following experiments were undertaken in which the type of carbohydrate was varied.

MATERIALS AND METHODS

Mice of the Princeton strain (2) were used exclusively. The composition of the purified diets is given in tables 1 to 3. The natural foodstuffs stock diet [3] contained 23% protein derived principally from skim milk powder, fish meal, and soybean oil meal; 4% fat; and about 60% carbohydrate, presumed to be mostly (cooked) starches. The pantothenic acid-deficient diet (designated 2517) was identical to the purified diet (2520), except for the omission of that vitamin.

All animals were fed ad libitum. They had access to the stock diet prior to complete weaning at 21 days, and were placed on experiment when males had attained a body weight of at least 14 g, and females 12 g, at about 4 weeks of age. At this time they were transferred from enamel pans containing pine shavings to wire-mesh-bottomed cages. For the first week, the purified diets were modified by mixing them with 25% of powdered stock diet to improve their acceptance and to reduce the number of spontaneous casualties

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[2] Presented in part at FASEB meeting, Atlantic City, New Jersey, April 16, 1969. Federation Proc., 28: 625, 1969 (abstract).
[3] Hemlock Hollow, Wayne, New Jersey.

TABLE 1

Composition of purified diets

	g/kg
Casein (vitamin free) [1]	180.0
Cottonseed oil	48.0
Glucose, sucrose, or raw cornstarch	709.0
Bone ash	25.0
Wesson salt mixture, modified [2]	12.0
Vitamin supplement [3]	20.0
1-Cystine	2.0
Choline-Cl	1.0
Cysteine-HCl (as antioxidant)	1.0
Tocopherol, carotene, and vitamin D_3 in cottonseed oil [4]	2.0

[1] Purchased from Whitson Products, New York, New York.
[2] Ingredients listed in table 2.
[3] Ingredients listed in table 3.
[4] Alpha-tocopherol acetate, 50 mg; vitamin A as carotene, 15,000 IU; vitamin D_3, 1000 IU.

TABLE 2

Composition of modified (3) *Wesson salt mixture*

	g/kg diet
NaCl	3.8
KCl	4.3
$MgSO_4$	3.3
$FeSO_4$ "dried"	0.57
	mg/kg diet
NaF	21.0
ZnO	12.0
$MnSO_4 \cdot H_2O$	16.1
Cu_2O	4.0
$AlK(SO_4)_2 \cdot 12H_2O$	3.3
KI	1.8

TABLE 3

Composition of vitamin supplement

	mg/kg diet
Thiamine·HCl	10.0
Pyridoxine·HCl	10.0
Riboflavin	20.0
Niacin	40.0
Calcium pantothenate [1]	40.0
Pteroyl glutamic acid	2.0
Biotin	0.20
Vitamin B_{12}	0.012
Inositol	200.0
Para-aminobenzoic acid	100.0

[1] Omitted from diet 2517.

during the first few days. Such deaths could generally be attributed to starvation. Thereafter, the experimental diets were fed undiluted. Both sexes were used, and were caged separately, five or six to a cage (18 by 20 by 20 cm).

In experiment 1, 53 mice were fed the complete purified diet, modified by substituting sucrose for glucose. There were 44 controls. In experiment 2, 61 mice received the diet prepared with cornstarch instead of glucose, and were compared with 57 controls. In experiment 3, 62 mice were fed the pantothenic acid-deficient diet, modified with cornstarch. Seventy controls received the glucose-containing pantothenate-deficient diet.

After 4 weeks on the undiluted diets, animals were anesthetized with ether, opened, and the duodenums were injected with buffered (pH 7.3) formalin via the stomach. Following this, the specimens were removed, opened and pinned out under additional formalin for completion of fixation and for observation. Lesions were measured with a micrometer eyepiece in a dissecting microscope.

RESULTS

Avillous hyperplasia. In the complete diet (2520), a highly significant reduction in incidence of lesions occurred as the complexity of dietary carbohydrates was increased (table 4). The average area of lesions was not significantly changed.

In the pantothenate-deficient diet (2517), when glucose was replaced by starch, the enhancing effect of the deficiency upon incidence and average area of lesions was eliminated for the former, and much reduced for the latter.

Fig. 1 Longitudinal section through the distal pylorus (right), and the proximal duodenum (left), of a pantothenate-deficient mouse. The large mucosal discontinuity in the center of the photograph is a deep chronic ulcer (U) which has penetrated all layers of the duodenum, and provoked marked edema, inflammation, and scarring of the underlying pancreas with its supporting connective tissue. To the left of the ulcer, the duodenal mucosa has lost nearly all of its customary villated character, and become thickened, due to marked overgrowth (hyperplasia) of the crypts of Lieberkühn (AH). The mucosa of the pylorus (PY) bears a superficial resemblance to the duodenal mucosa, at this magnification, but is actually not involved in the disease process. Chlorazole black E. × 48. (Photograph by Leo Goodman, Mallory Institute of Pathology.)

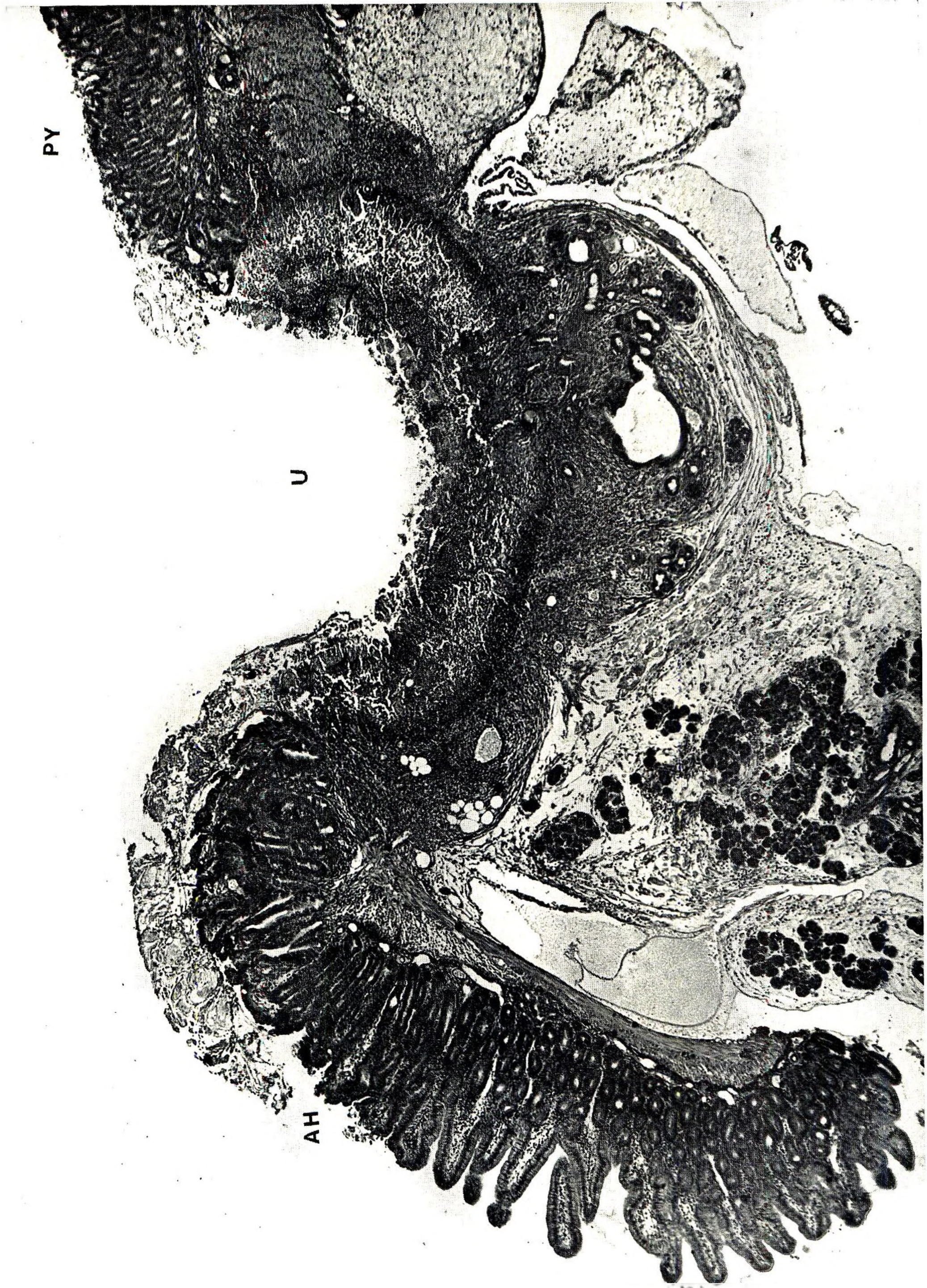

Figure 1

TABLE 4

Incidence and area of lesions; complete diets (2520), and pantothenate-deficient diets (2517)

Diet	Incidence	Area				
		Avg [1]	SE	df	t	P
	%	mm^2				
2520 (Glucose)	91.0	2.48	0.54	—	—	—
2520 (Sucrose)	56.7	3.42	0.76	—	—	—
Comparison	$\chi^2 = 14.093$	—	—	68	1.038	0.4–0.2
2520 (Glucose)	86.0	2.48	0.86	—	—	—
2520 (Starch)	11.5	1.29	0.43	—	—	—
Comparison	$\chi^2 = 65.575$	—	—	54	0.983	0.4–0.2
2517 (Glucose)	97.0	16.38	1.29	—	—	—
2517 (Starch)	82.0	6.25	0.94	—	—	—
Comparison	$\chi^2 = 8.213$	—	—	117	6.012	< 0.001

[1] Averages include only affected animals.

Ulcer. The incidence of chronic duodenal ulcer in mice fed the glucose-containing pantothenate-deficient diet was 30%. When cornstarch was substituted for glucose, the incidence fell to 18%, but this was not statistically significant ($\chi^2 = 2.71$). When figures in the control series were supplemented with 56 additional cases taken from previous experiments in which the cornstarch modification had not been fed, the value for χ^2 rose to 2.95—still remaining below the level of significance.

DISCUSSION

Focal avillous hyperplasia of the proximal duodenal mucosa is shown to be affected by the type of carbohydrate ingested. An influence of this sort has not, to our knowledge, been previously reported, and the mechanism is unknown. Logically, we presume it to be related, directly or indirectly, to the nature of the chyme whose composition would vary according to whether a starch or glucose diet had been fed. In the former instance it is unlikely that more than traces of glucose would be present in the proximal duodenum, while distally, rapid absorption of the glucose gradually released by digestion, would prevent any marked accumulation (4). In the case of a high-glucose diet, an abundant and relatively concentrated glucose solution would strike the duodenal mucosa, affecting it perhaps directly through its pH or osmotic properties, or indirectly by fostering abnormal bacterial or protozoan inhabitants. Differential metabolic effects produced by the feeding of various carbohydrates are abundantly documented (5), but we have found no reported link between any of these and the morphology of the intestinal mucosa.

Avillous hyperplasia closely resembles a nonspecific lesion which has been encountered clinically and experimentally under a variety of conditions (6). In their descriptions, investigators generally have put greater emphasis upon the absence of villi than upon subvillous changes; and the lesion has been most frequently referred to as "villus atrophy." Because villus cells are passive as far as growth is concerned, and because the crypts from which they arise are so manifestly altered, we feel that our emphasis is justified. In the course of the present investigation, a carbohydrate effect has been recognized, and it has become possible to separate it from that of pantothenic acid deficiency. This removes the carbohydrate effect, at least provisionally, from our main line of investigation of chronic duodenal ulceration, although the phenomenon remains of interest in itself.

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Vitamin E Deficiency and Fat Stress in the Dog [1,2]

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ABSTRACT Thirty-two male beagle puppies were fed vitamin E-deficient diets with four levels (1, 5, 10, and 15%) of safflower oil with or without a vitamin E supplement for a 15-week period. The unsupplemented dogs developed a vitamin E deficiency which was correlated with increased dialuric acid hemolysis of red cells and decreased plasma tocopherol values. Both hemoglobin and packed cell volume were depressed by increasing fat consumption, unrelated to tocopherol supplementation and attributed to in vivo red cell disruption. Creatine phosphokinase values were elevated in tocopherol-deficient dogs and were correlated with fat consumption. Terminal plasma vitamin A concentrations were lower in dogs receiving more than 1% supplementary fat. At necropsy, browning of the intestinal muscularis in the tocopherol-deficient dogs was related to the consumption of polyunsaturated fats (PUFA). Microscopically, lipofuscin was seen in smooth muscle of gut, urinary bladder and small arterioles. Neuroaxonal dystrophy and myodegeneration were also found in the vitamin E-deficient dogs. The requirement for tocopherol was directly related to PUFA consumption, apparently associated with the metabolism of the fat and not with an antioxidant role of the vitamin.

Reports of a syndrome in the dog, suggestive of vitamin E deficiency, have appeared in the literature during the past 50 years. These include brown pigmentation of the intestinal muscularis (1–4), myodegeneration (5–7), testicular degeneration (6), and other reproductive disorders (3–5). The clinical and necropsy findings from several dogs at a kennel, where large quantities of polyunsaturated fats (PUFA) apparently produced a tocopherol deficiency (3), provided the basis for the present experimental study of vitamin E deficiency in this species. In addition, the comparative aspects of the brown bowel syndrome in man (8) and the recent emphasis on human vitamin E deficiency (9, 10) added further impetus.

EXPERIMENTAL

Thirty-two weanling, male beagle puppies, 7.5 to 9.5 weeks old and weighing 4.0 ± 1.1 pounds, were obtained from a commercial dog farm in four lots of eight. Each puppy was kept in an individual pen with 1.5 square meters of concrete floor space, bedded with sawdust. Incandescent light was provided for 12 hours per day and the temperature was maintained above a minimum of 16°.

For a 15-week comparison period, each pup in each replicate received one of four vitamin E-low diets containing 1, 5, 10, or 15% stripped safflower oil [4] (SFO) as a source of polyunsaturated fat (table 1) and no added tocopherol (deficient) or plus added tocopherol (adequate). The diets (table 1), mixed every 10 days, were formulated according to National Research Council (NRC) requirements (11) such that protein content per 100 calories was essentially constant, fat replacing carbohydrate with increasing fat content. Vitamins, other than tocopherol, were premixed and refrigerated until their required use. Due to the differences in the calculated mineral contents of the four diets, separate mineral mixes were employed for each diet to meet the NRC requirement (11). Tocopherol [5] (11 mg/kg live weight) was added to each puppy's diet at the once-a-day feeding for those animals (adequate) designated to receive it. The feed allotment was adjusted weekly to meet NRC caloric requirements for the growing pup (11). At each level of dietary fat and within each replicate, the

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[3] Present address: Department of Nutrition, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115.
[4] Obtained through the courtesy of Dr. R. H. Bunnell, Hoffmann-La Roche, Inc., Nutley, New Jersey.
[5] D-α-Tocopherol acetate, Myvamix, Distillation Products Industries, Rochester, New York.

TABLE 1

Basal rations [1]

Ingredients	1	2	3	4
		kg/100 kg		
Toasted cornflake crumbs	53.8	48.1	39.0	30.9
White corn meal [2]	10.0	10.0	10.0	10.0
Fish meal [3]	12.5	13.5	14.0	15.0
Casein	8.0	9.0	12.5	15.0
Dried brewer's yeast	3.0	3.0	3.0	3.0
Dried skim milk	5.0	5.0	5.0	5.0
Cerelose	3.5	3.5	3.5	3.5
Safflower oil [4]	1.0	5.0	10.0	15.0
Iodized salt	0.5	0.5	0.5	0.5
Mineral mixture [5]	2.6	2.3	2.4	2.0
Vitamin mixture [6]	0.1	0.1	0.1	0.1
Calories (digestible/g)	2.75	2.91	3.14	3.38

[1] With the exception of tocopherol, these rations meet the NRC nutrient concentration requirements for growing puppies (table 2, NAS-NRC, 989, 1962) and were based upon feed composition tables (NAS-NRC, 1232, 1964).

[2] Degermed, debranned white corn meal (Quaker Oats Company).

[3] Viobin process, New Bedford Fish Products Company, New Bedford, Mass.

[4] Stripped of tocopherols and contained 0.32 mg/100 ml alpha-tocopherol.

[5] Contributed the following minerals from reagent grade sources per 100 kg of ration: (in grams) *Diet 1*: Ca, 200.2; Co, 0.22; Cu, 0.33; K, 349.8; Mg, 279.4; P, 294.8. *Diet 2*: Ca, 123.2; Co, 0.22; Cu, 0.33; K, 349.8; Mg, 289.3; P, 234.3. *Diet 3*: Ca, 100.1; Co, 0.22; Cu, 0.33; K, 400.4; Mg, 309.1; P, 237.6. *Diet 4*: Ca, 0.0; Co, 0.22; Cu, 0.33; K, 399.3; Mg, 320.1; P, 158.4.

[6] Contributed the following vitamins per 100 kg of ration: vitamin A acetate (10,000 IU/g), 44 g; vitamin D_3, (3,000 IU/g), 11 g; menadione, 11 mg; cyanocobalamin (1 mg/g), 1.1 g; choline chloride (92%), 29.7 g.

pup receiving the tocopherol was fed the same amount of feed per unit of live weight as the puppy receiving no tocopherol.

The puppies were weighed weekly and examined daily. They were treated for intestinal nematodes prior to and during the comparison period and vaccinated for distemper, hepatitis, and leptospirosis at 12 weeks of age.

Blood analyses. A 15-ml heparinized, fasting blood sample was drawn from the jugular vein at zero, 5, 10, and 15 weeks. Six drops of blood were placed in a 5-ml saline-phosphate buffer for the dialuric acid hemolysis test (12) and 2 to 3 ml placed in a small vial for hemoglobin (13) and microhematocrit (14) analyses. With the plasma obtained from the remainder of the sample, plasma tocopherol (15), creatine phosphokinase (CPK) (16), plasma glutamic-oxalacetic transaminase (SGOT) and plasma glutamic-pyruvic transaminase (SGPT) (17) were determined. In addition, terminal plasma vitamin A (18) and ascorbic acid (19) were determined from additional blood collected at the 15-week sampling.

Necropsy procedures. Following the last bleeding, all eight dogs in each replicate were killed by intravenous injection of sodium pentobarbital and necropsied in pairs, i.e., both the adequate and its deficient pair mate fed the same %-fat diet. All organ systems were inspected grossly and representative sections of each fixed in 10% buffered formalin. The eyes were fixed in Zenker's solution, and bone marrow smears were made and stained with Wright-Giemsa stain.

Color transparencies were taken of a 6-inch segment of the ileum, removed at a point 10 inches anterior to the cecum in each dog. A number gradation from 0 to 10 was employed to quantitate the degree of color development seen in the ileum at necropsy, in color photographs, and in the histologic sections stained with hematoxylin and eosin.

All sections were routinely processed and stained with hematoxylin and eosin. In addition, representative paraffin sections were stained with periodic acid-Schiff (PAS), Masson's trichrome, Ziehl-Neelsen acid fast, and Schmorl's ferri-ferrocyanide. Formalin-fixed frozen sections of intestine and skeletal muscle were also stained with oil red O and Sudan black (20, 21).

The statistical procedures were those of Cochran and Cox (22).

RESULTS

During the 15-week comparison period, feed consumption and growth were comparable for all dietary treatments (table 2). Two dogs on high fat diets (15%, −E and 10%, +E) in the second replicate developed severe diarrhea during week 2 of the comparison period. One of these (15%, −E) died, while the second recovered after fluid and antibiotic therapy and was restored to treatment within 10 days. A dry cough, seemingly precipitated by the stress of the week-5 bleeding, persisted throughout the experiment, although antibiotic therapy appeared to reduce its evidence.

Blood analyses. Initial plasma tocopherol values for all puppies ranged from 157 to 371 μg/100 ml (average 260), whereas terminal comparison period values ranged from 0 to 127 μg/100 ml (average 51) in the deficient dogs and from 576 to 1936 μg/100 ml (average 1413) in the supplemented animals. There was a tendency for a negative trend in terminal values with increasing levels of dietary fat for the deficient dogs and a corresponding positive trend in the tocopherol-supplemented animals, but neither was significant (table 3).

The red blood cells of all puppies were hemolyzed in varying degrees by dialuric acid at the initial bleeding. The percentage hemolysis of the red cells was reduced to zero in subsequent bleedings for the tocopherol-supplemented dogs, while for deficient puppies it increased throughout the comparison period and was linearly related to percentage dietary fat ($P < 0.05$) for the terminal values (table 3).

Although hemoglobin and packed cell volume increased above initial values, the terminal values were significantly depressed ($P < 0.05$) by increased fat consumption. This trend was independent of tocopherol supplementation (table 4).

The comparison-period average and terminal SGOT (table 5) were higher than the initial values for all dogs; however, comparison-period average activities of the dogs fed supplements of tocopherol decreased with higher levels of dietary fat ($P < 0.05$). This was also the case for the terminal measurement ($P < 0.10$.). The increased values for the unsupplemented dogs were not significant, although the terminal value for the 10%-fat, unsupplemented dogs was noticeably elevated.

SGPT values (table 5) did not change appreciably during the 15-week comparison period. Dogs fed no supplemental tocopherol had slightly elevated comparison-period average values which correlated with an increase in the dietary fat percentage ($P < 0.02$).

CPK (table 5) increased significantly during the comparison period in all dogs. In those dogs fed no supplemental tocopherol, the comparison-period average values increased significantly ($P < 0.01$) as the percentage of dietary fat was increased. The terminal values for the same dogs showed a similar trend ($P < 0.10$).

There were no differences in terminal plasma ascorbic acid levels due to any treatment (table 5). Terminal levels of plasma vitamin A were lower in the groups supplemented with more than 1% fat, (table 5).

Morphologic changes. Observations at necropsy included pigmentation and contraction of the intestinal muscularis. The brown discoloration of the serosal surface was seen in tocopherol-deficient dogs only and extended from the pyloric region of the stomach to the midportion of the colon (fig. 1). It appeared most intense in the jejunum and proximal ileum, the degree of pigmentation being directly related to the levels of dietary fat (table 6). With prolonged exposure to the air, the pigment darkened considerably and the intestinal wall contracted. The degree of contraction was generally correlated with fat consumption, although the pigmented intestine of tocopherol-deficient dogs seemed to be most severely affected.

Histologically the pigment was most prominent in the smooth muscle cells of the ileum and was distributed throughout the internal and external muscularis, being slightly more intense in the former. It was evident as small perinuclear granules that occasionally formed large aggregates (fig. 2). In heavily pigmented cells, the nucleus was disrupted and granules were scattered throughout the cytoplasm. Vacuolization of the nucleus and perinuclear region charac-

TABLE 2

Comparison-period data on number of dogs, length of comparison period, initial age, average feed and caloric consumption, live weight gain, and feed efficiency

Criterion	Diets: Tocopherol (−) % Fat				Diets: Tocopherol (+) % Fat				SD per dog
	1	5	10	15	1	5	10	15	
No. dogs per treatment	4	4	4	3	4	4	4	4	—
Comparison period, weeks	15	15	15	15	15	15	15	15	—
Initial age, weeks	8.4	8.5	8.6	8.4	8.5	8.2	8.2	8.2	0.9
Feed consumption, avg grams/day									
fed	254	214	248	217 [1]	214	216	194	181	33
consumed	246	206	240	204 [1]	210	212	194	172	29
Caloric consumption, avg cal/day									
fed	699	623	780	735 [1]	588	629	611	611	100
consumed	677	598	752	692 [1]	577	617	611	582	86
Live wt									
initial wt, kg	1.95	1.72	2.05	2.11 [1]	1.72	1.66	1.64	1.64	0.38
gain, avg grams/day	64	57	68	62 [1]	54	59	60	60	10
Feed efficiency, cal/g gain	10.8	10.6	11.1	11.2 [1]	10.8	10.5	10.1	10.4	1.0

[1] Includes one calculated missing value (22).

TABLE 3

Initial, comparison-period average, and terminal data for plasma levels of tocopherol and dialuric acid hemolysis

Criterion	Diets: Tocopherol (−) % Fat 1	5	10	15	Tocopherol (+) % Fat 1	5	10	15	SD per dog
Plasma tocopherol, µg/100 ml									
Initial									
actual	296	246	249	299	282	256	259	266	—
log_{10}	2.46	2.37	2.39	2.45 [1]	2.45	2.39	2.41	2.41	0.07
Average (15 wk)									
actual	123	115	100	107	928	921	1070	1124	—
log_{10}	2.08	2.05	1.99	2.01 [1]	2.95	2.96	3.02	3.05	0.08
Terminal									
actual	64	64	36	41	1195	1450	1500	1508	—
log_{10}	1.68	1.72	1.40	1.53 [1]	3.04	3.15	3.16	3.17	0.25
Dialuric acid hemolysis, %									
Initial									
actual	44	61	57	54	76	64	78	59	—
arcsin, %	41	52	50	48 [1]	61	54	62	52	10
Average (15 wk)									
actual	72	79	79	78	19	16	20	15	—
arcsin, %									
unadj	58	63	63	63 [1]	26	24	26	22	4
adj [2]	63	63	64	64 [1]	22	23	23	23	2
Terminal									
actual	74	81	87	88	0	0	0	0	—
arcsin, %	60	65	70	74 [1]	0	0	1	1	7

[1] Includes one calculated missing value (22).
[2] Adjusted by analysis of covariance for initial values (22).

TABLE 4

Initial, comparison-period average, and terminal values for hemoglobin and packed cell volume

Criterion	Diets: Tocopherol (−) % Fat 1	5	10	15 [1]	Tocopherol (+) % Fat 1	5	10	15	SD per dog
Hemoglobin, g/100 ml									
Initial	8.9	8.6	9.1	8.8	8.5	8.3	8.9	8.0	0.8
Average (15 wk)									
unadj	9.5	8.9	8.9	8.3	9.4	8.5	8.9	8.0	0.9
adj [2]	9.3	9.0	8.5	8.1	9.4	8.8	8.7	8.6	0.7
Terminal									
unadj	11.1	10.2	10.0	8.9	10.7	9.5	9.8	8.8	1.3
adj	10.8	10.3	9.6	8.8	10.8	9.8	9.6	9.3	1.1
Packed cell volume, %									
Initial	30.1	28.6	30.0	28.2	28.8	26.9	24.4	27.0	2.6
Average (15 wk)									
unadj	30.6	29.8	29.5	28.0	30.7	28.8	30.0	27.2	2.4
adj [2]	29.7	29.8	28.6	28.2	30.6	29.9	29.6	28.2	1.8
Terminal	32.1	34.4	31.4	29.8	34.1	31.5	31.9	28.1	3.3

[1] All entries in this column include one calculated missing value (22).
[2] Adjusted by analysis of covariance for initial values (22).

TABLE 5

Initial, comparison-period average, and terminal data for plasma transaminases and creatine-phosphokinase, and terminal plasma vitamin A and ascorbic acid

Criterion	Diets: Tocopherol (−) % Fat				Tocopherol (+) % Fat				SD per dog
	1	5	10	15	1	5	10	15	
Transaminases, Sigma-Frankel Units [1]									
Glutamic-oxaloacetic [2]									
initial	15	13	16	17	15	14	11	11	2
average (15 wk)	20	16	25	22	23	23	18	16	3
terminal	19	13	40	16	29	28	20	15	8
Glutamic-pyruvic									
initial	17	14	12	14 [3]	15	14	11	12	5
average (15 wk)									
unadj	15	13	15	22 [3]	14	13	12	11	5
adj	13	13	16	22 [3]	14	13	13	11	5
terminal	16	14	25	13 [3]	14	13	12	11	6
Creatine-phosphokinase, Sigma Units [4]									
initial	5	5	6	6 [3]	5	11	7	12	5
average (15 wk)	10	18	19	41 [3]	15	12	10	16	10
terminal	16	20	43	30 [3]	31	18	13	28	12
Plasma vitamin A, μg/100 ml									
terminal	136	110	95	99 [3]	112	102	92	97	16
Plasma ascorbic acid, mg/100 ml									
terminal	0.86	0.82	0.86	0.76 [3]	0.96	0.80	0.85	0.93	0.12

[1] A Sigma-Frankel Unit is a measure of the enzymatic activity that will form 4.82×10^{-4} M glutamate/minute at a pH of 7.5 and at 25° resulting in a decrease in the optical density at 340 mμ of 0.001/min per ml per centimeter of light path of serum.
[2] Based on two dogs per dietary treatment.
[3] Includes one calculated missing value (22).
[4] Based on three dogs per dietary treatment. One Sigma Unit of CPK will phosphorylate 1 mμmole creatine per minute at 25° under the conditions of Sigma Procedure No. 40-UV (dated September 1965 or later).

terized the less affected cells and was occasionally the only visible change in hematoxylin and eosin sections, the granules being discernible only after PAS staining. In addition to being PAS positive, the granules stained with Schmorl's iron reduction and Sudan black. They were oil red O negative and only the largest, oldest aggregates were acid fast. In PAS-stained sections from tocopherol-supplemented dogs, light to moderate pigmentation was also present, especially at the high levels of fat intake. In these sections, the pigment was predominantly located in the internal muscularis adjacent to the submucosa.

The contraction of the gut that developed post mortem was seen histologically as bizarre striations in five dogs, four deficient and one adequate, the latter receiving the 15%-fat diet. In hematoxylin and eosin sections the striae appeared as alternating bands of eosinophilic cytoplasm and clear "ghost cell" portions of the smooth muscle cell, suggesting that myofibrils had broken their attachments with the sarcolemma and contracted into dense cytoplasmic regions. Nuclei situated in the contracting cytoplasm were also condensed and intensely basophilic (fig. 4). Similar contraction was seen in the bladder of five deficient dogs. Extensive nuclear vacuolization and pigment accumulation were also seen in the bladder smooth muscle of tocopherol-deficient dogs.

Smooth muscle of small arterioles in sections of degenerating skeletal muscle showed vacuolization of peripheral cells

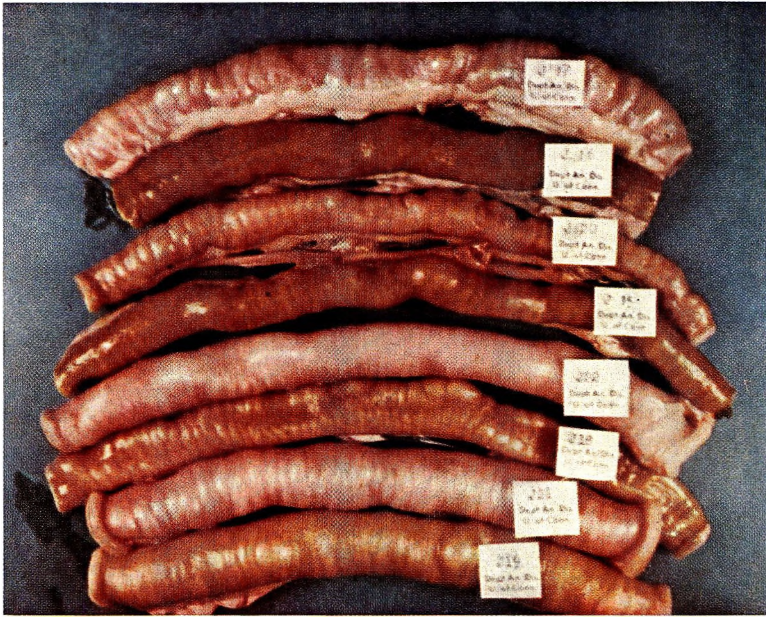

Fig. 1 Section of ileum from each dog in first replicate. Notice the increased pigmentation with increased fat consumption in the tocopherol-unsupplemented dogs and the marked contraction with increased fat intake, especially in the pigmented gut. From bottom: 1% fat-deficient, control; 5% fat-deficient, control; 10% fat-deficient, control; 15% fat-deficient, control.

TABLE 6

Degree of pigmentation of the proximal ileum of dogs fed no supplemental tocopherol, recorded at necrospy, from color transparencies taken 2 to 8 hours post mortem, and from hematoxylin and eosin sections (scale 0 to 10)

Replicate	At necropsy % Dietary fat				From transparencies % Dietary fat				From H and E sections % Dietary fat			
	1	5	10	15	1	5	10	15	1	5	10	15
1	1	3	5	7	3	5	7	9	4	6	9	9
2	2	5	5	— [1]	5	8	8	—	3	6	3	—
3	2	1	3	7	5	4	7	9	1	1	3	6
4	1	5	7	3	2	2	6	4	1	1	6	2
Average	1.5	3.5	5.0	5.7	3.8	4.8	7.0	7.3	2.2	3.5	5.2	5.7

[1] Died during experimental week 2.

and very slight pigment accumulation. Other smooth muscles in the spleen, lung, and bile duct had traces of pigment but were not objectively characterized.

Degenerative lesions of skeletal muscle were found in the tocopherol-deficient dogs in the superficial pectoral, semimembranosus, biceps femoris, psoas, tongue, and diaphragm, approximately in that order. The degeneration was typical of that described for other vitamin E-deficient animals and was characterized by swelling and hyalin necrosis of individual muscle fibers (fig. 3). These lesions were not extensive, although there was moderate involvement in high-fat dogs on deficient diets. In formalin-fixed frozen cross sections stained with oil red O, the red and white fiber types could be distinguished by the numerous lipid droplets associated with the mitochondria in the red type. In sections of muscle from tocopherol-defici-

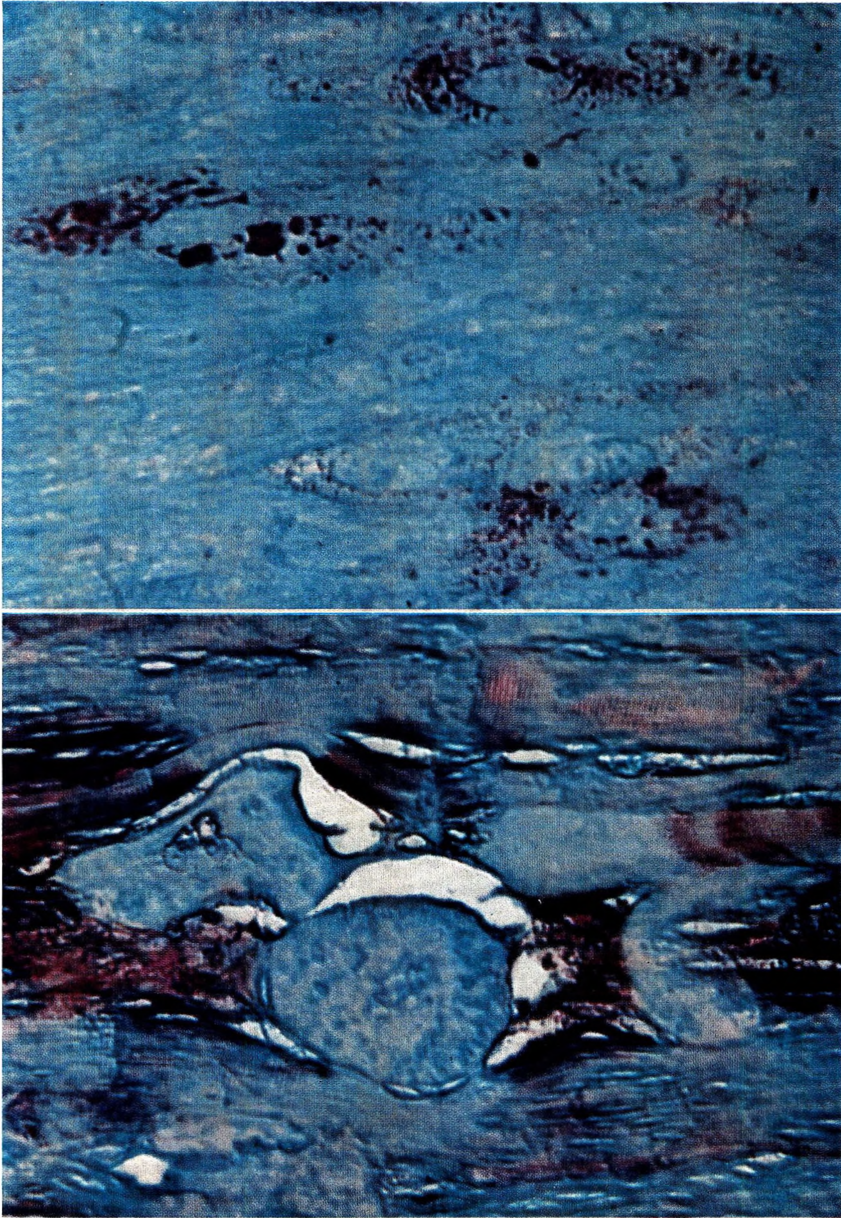

Fig. 2 (upper) Internal muscularis from 15% fat, tocopherol-unsupplemented dog. The perinuclear, PAS-positive lipofuscin granules are apparent. PAS-light green × 1000.

Fig. 3 (lower) An acute, but not extensive, degeneration of skeletal muscle was present in many of the tocopherol-deficient dogs. Here, a segment of a fiber is interrupted by Zenker's necrosis. PAS-light green × 100.

ent dogs, early signs of degeneration were recognized by large swollen fibers. These degenerating fibers did not contain lipid and appeared to be white fiber types (fig. 5).

Another alteration of muscle related to high fat consumption, but more pronounced in tocopherol-deficient dogs, was the replacement of muscle fibers by fat.

Fig. 4 These bizarre striations were seen in the smooth muscle of intestine and urinary bladder of five high-fat, tocopherol-deficient dogs and thought to be an artifact of postmortem hydroperoxides. H & E × 94.

The fat appeared to be displacing sarcoplasm, but its interstitial location could not be disproved (fig. 6).

Changes in the central nervous system were restricted to a degeneration of axons, especially noticeable in the *n. gracilis* and *n. cuneatus* of the brain stem. This alteration was found only in tocopherol-deficient animals and was again more pronounced at high fat levels (fig. 7). These dystrophic axons ranged in size from approximately 30 to 150 μ. The smaller spheroids were dense, eosinophilic and occasionally ringed by a dark red fragmented collar, presumably the myelin sheath. The larger variety were irregular masses of homogeneous, eosinophilic material often with small granular particles within their margins. The largest were pale, swollen masses that appeared to have degenerated beyond the point of staining affinity. The small bodies were strongly PAS positive and stained a deep orange-brown with Masson's trichrome. The larger bodies did not stain with either technique.

The spleens contained excessive amounts of hemosiderin, and bone marrow counts were within normal myeloid–erythroid (M/E) ratios for dogs (14), approximately 1.6 to 2.0.

Due to the immaturity of the dogs, no treatment differences could be ascertained for the testes. All dogs with maturing spermatocytes showed some degree of nuclear pyknosis and vacuolization. The tocopherol-deficient animals appeared to have excessive fatty vacuoles in the seminiferous tubules, but this was inconsistent.

DISCUSSION

The vitamin E deficiency that developed in the unsupplemented dogs was substantiated by the low plasma tocopherol values and the in vitro hemolysis of red blood cells,

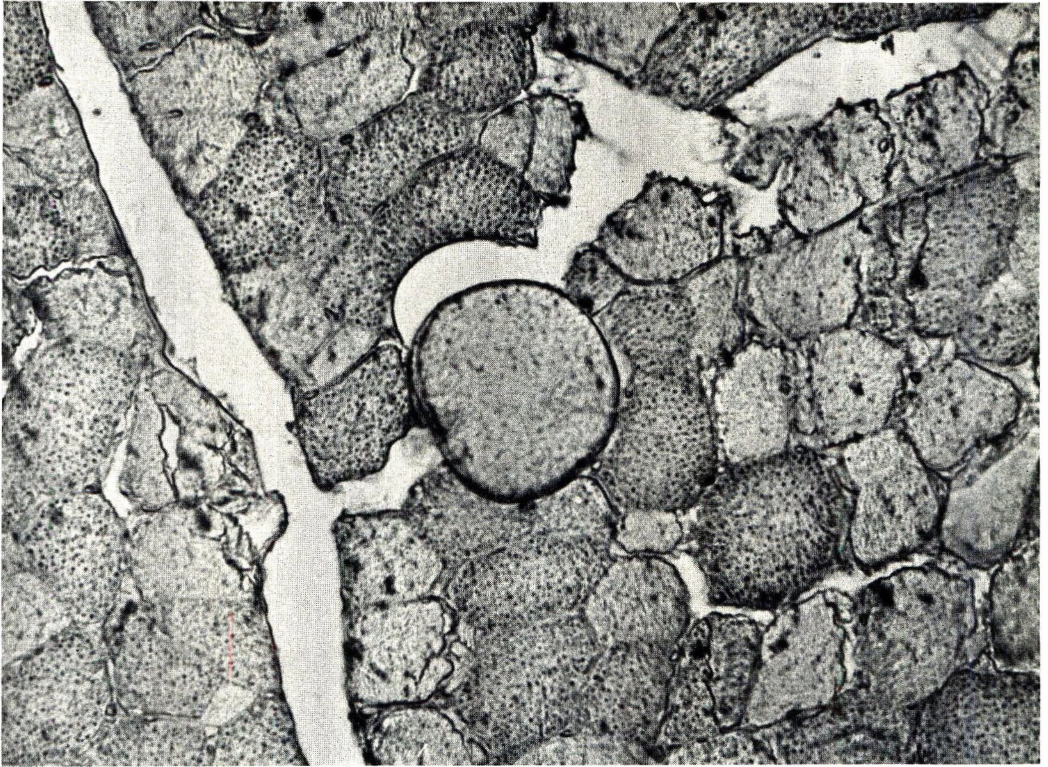

Fig. 5 Formalin-fixed frozen section of the semimembranosus muscle from a tocopherol-deficient dog. Red and white fibers can be distinguished by the lipid droplets associated with the mitochondria of the red type. Early degeneration, characterized by swollen fiber (center), was seen in numerous, apparently white, fibers Oil red O $\times$ 94.

considered an early indication of this vitamin deficiency (23). The positive trend in plasma tocopherol concentration with increased dietary fat in the tocopherol-supplemented dogs was interpreted as increased absorption of the vitamin associated with higher fat absorption (24). The corresponding negative trend in the tocopherol-unsupplemented dogs was interpreted as in vitro destruction of any available tocopherol in the intestine by the peroxides associated with diets high in PUFA and the increased utilization of in vivo tocopherol associated with increased metabolism of PUFA.

The positive correlation between percentage dialuric acid hemolysis and fat intake of the deficient dogs is evidence of the probable in vitro antioxidant potential of tocopherol. The greater hemolysis at high dietary fat levels might reflect an increased incorporation of PUFA into the red cell membrane (25), which would enhance the possibility of in vitro peroxidation. This tendency for dietary PUFA to increase hemolysis, previously demonstrated in rats (26), might also reflect a structural instability introduced by excessive PUFA in the cell membrane (27). Furthermore, the higher fat tocopherol-unsupplemented diets tended to result in lower plasma tocopherol values, suggesting an additive effect.

Similarly, the depression in hemoglobin and packed cell volume produced by high dietary fat was probably related to an increased susceptibility of the red cell to in vivo hemolysis. It has been demonstrated in the dog (28), rat (29), and mink (30) that high fat diets, irrespective of tocopheral status, have a depressing effect on packed cell volume. In the dog this was attributed to increased hemolysis enhanced

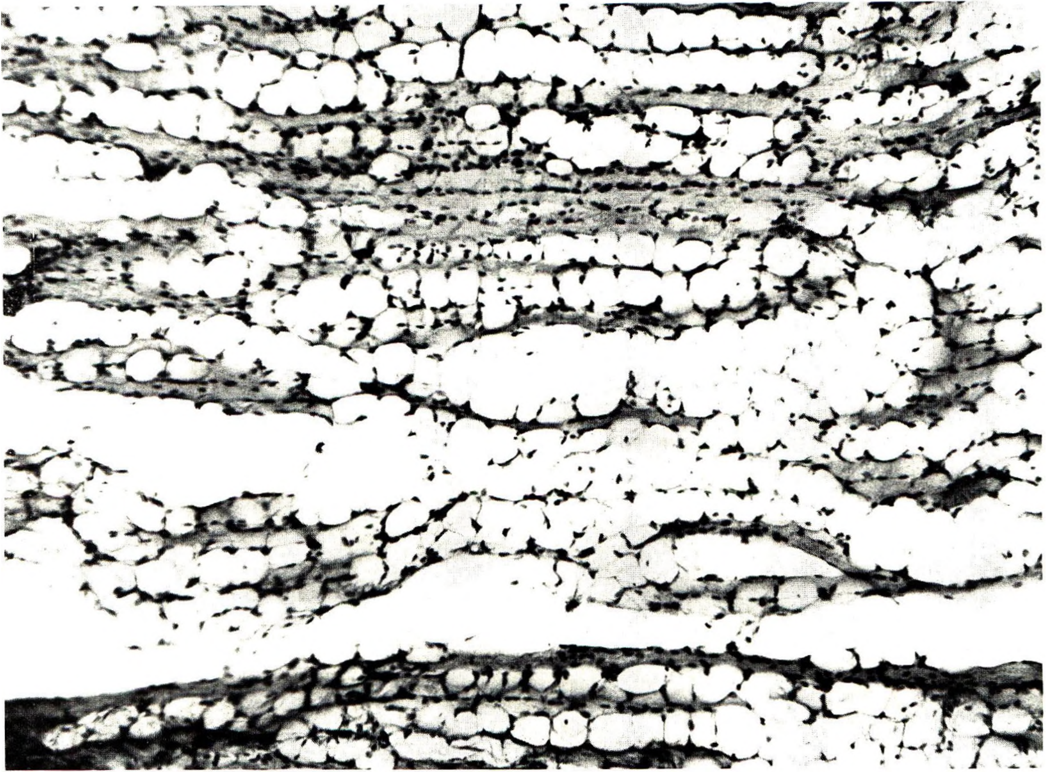

Fig. 6 Fatty replacement of muscle seemed to be associated with high fat consumption, but was more pronounced in tocopherol-deficient dogs. Tongue. H & E × 60.

by the lytic action of fatty acids (28). The hemosiderosis of the spleens provided evidence of a hemolytic phenomenon. The bone marrow apparently continued to produce red blood cells at a normal rate, as the M/E ratio was not markedly changed. In none of the marrow smears was there evidence of binucleate megaloblasts described in other vitamin E-deficient species (31–33).

Since significantly elevated terminal values for CPK, but not SGOT, were observed in the higher-fat-intake tocopherol-unsupplemented dogs, the former measurement was a better indicator of the mild myodegeneration present in these dogs. The trend toward an elevated SGPT during the comparison period for this same group of dogs may indicate slight damage to liver cells.

Plasma ascorbic acid was not influenced by treatment, which is of interest in light of the known depression of ascorbic acid synthesis by the liver of the vitamin E-deficient rat and rabbit (34). Terminal plasma vitamin A concentrations were lower in dogs receiving more than 1% supplementary fat.

The pigment granules of the intestinal muscularis, originally deemed unclassifiable and called "leiomyometaplasts" (35), but classified herein and elsewhere as a lipofuscin (3, 36), directly reflected the amount of fat consumed by the tocopherol-unsupplemented dogs. The darkening of the pigment on exposure to air presumably results from rapid atmospheric peroxidation of the perinuclear lipoprotein seen histologically. Related to this was the bizarre contraction of smooth muscle, which could result from postmortem peroxides of PUFA, demonstrated to induce extreme contraction of smooth muscle in isolated guinea pig ileum (37).

Since graded levels of tocopherol were not fed in this experiment, little can be

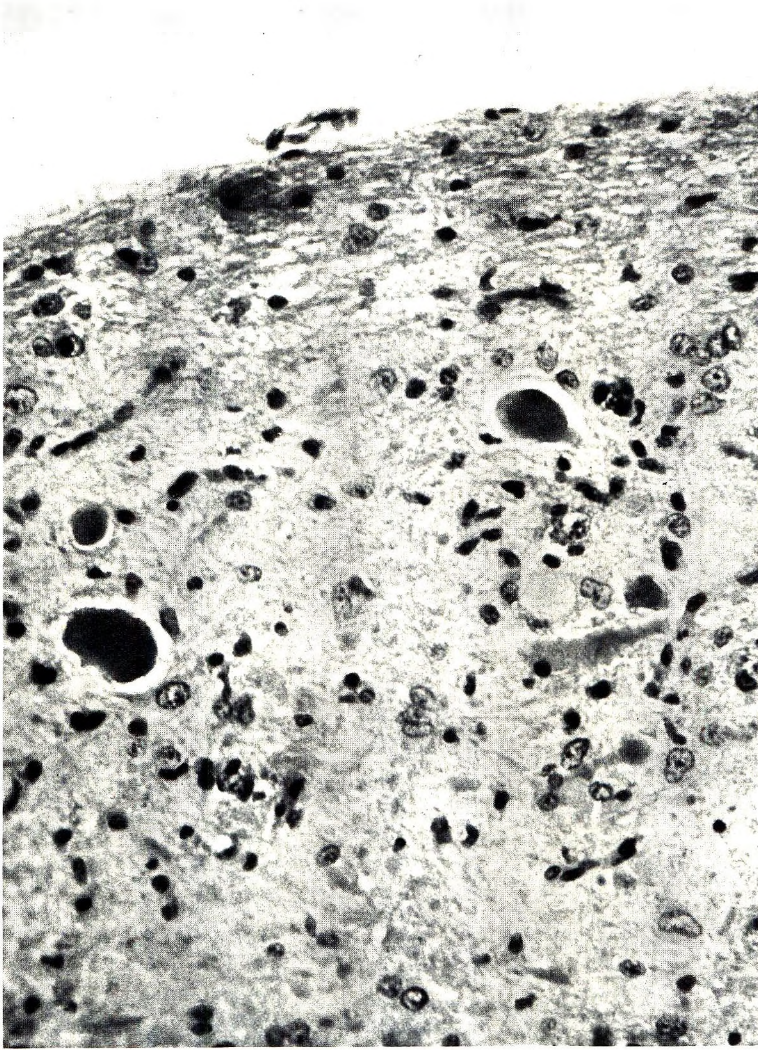

Fig. 7 Numerous dystrophic axons were visible in the *n. gracilis* (photo) and *n. cuneatus* of the tocopherol-deficient dogs. H & E × 94.

concluded regarding the growing pup's requirement for tocopherol. The high level of daily tocopherol supplementation (11 mg/kg body wt per day) was intended to assure that complete tocopherol requirements would be met for all levels of dietary fat stress. In every case of the tocopherol–PUFA ratio (mg/g) was greater than 2.0. Since 0.6 has been suggested as the necessary ratio for antioxidant protection of dietary PUFA (38), these dogs must be considered adequately protected. Because most of the supplemented dogs had traces of lipofuscin in their intestinal muscularis, which appeared to correlate with fat consumption, some doubt is cast on the reliability of a simple "antioxidant/unit fat" relationship as a measure of the vitamin E requirement. An antioxidant function was not suggested by an experiment in which high levels of vitamin E were unable to prevent ceroid formation in livers of rats fed a low protein diet supplemented with corn oil (39).

Similarly, the results of recent experiments specifically designed to test the in

vivo antioxidant activity of tocopherol have been consistently negative (40). Pigment formation may be the result of an accumulation and polymerization of PUFA due to an overload of these lipids on the metabolism of the smooth muscle cell. Thus, the problem would be related to lipid metabolism and not to the antioxidant capacity of this vitamin. If tocopherol is truly lipotropic, directly or indirectly and unrelated to its antioxidant potential, and the pigment formation deemed a consequence of defective lipid metabolism, then the presence of pigment can be explained both in the absence of tocopherol (inadequate lipotropism with peroxidized polymerization of accumulated PUFA-lipoprotein) or in the presence of tocopherol with an excess of polymerizable PUFA (cellular overload).

The alterations of smooth muscle seen in small arterioles reemphasizes the effect of the high-PUFA, vitamin E-deficient diet on the smooth muscle cell. Pigment accumulation in the wall of small arteries has been noted as a prominent feature in clinical cases of suspected tocopherol deficiency, both in dogs (3) and in man (41). Also, the potential of lipid peroxides in the genesis of atherosclerosis has been experimentally substantiated (42). A diet similar to that fed to these dogs has produced a lesion in pigs, particularly in myocardial vessels, that was characterized as a microangiopathy (43), and it has been concluded by others studying the vitamin E-deficient monkey (44) and the chick with encephalomalacia (45) or exudative diathesis (46) that vascular lesions are a prominent feature of the morphologic changes.

Dystrophic axons are not uncommon in older dogs and have been characterized (47). They have received considerable attention in man (48), and their association with vitamin E deficiency is well established in the rat where the degeneration of axons is thought to be responsible for the posterior ataxia and paralysis seen in prolonged tocopherol deficiency in that animal (49). In an electron microscopic study, Lampert et al. (49) demonstrated mitochondrial proliferation and numerous electron-dense bodies, supposedly lipid granules, which appear similar to the lipofuscins in the smooth muscles of the rat uterus (50) and the dog intestine.[6]

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Effect of Hypophysectomy on Lipid Composition in the Immature Rat[1]

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ABSTRACT Studies of the effect of hypophysectomy on the composition of the lipids of hearts, kidneys, livers, epididymal fat pad and testes of immature rats of the Sprague-Dawley strain are reported. Hypophysectomized weanling rats (3 weeks of age) were killed in groups of six at selected intervals over a 44-week period and compared to normal animals of the same age fed the same diet. Feed consumption, growth and weights of organs of both hypophysectomized and normal animals were measured. The hypophysectomized animals grew to only about one-third the size of normal animals. The hearts, livers, kidneys and epididymal fat pads of the hypophysectomized animals consisted of approximately the same percentage of the body weight as those of the normal animals. In contrast, the testes of the hypophysectomized animals were only about one-tenth the weight of those of the normal animals. After 18 weeks, most of the hypophysectomized animals exhibited shaggy coats, scaly tails and feet, characteristic of the dermal symptoms of an essential fatty acid (EFA) deficiency. The effect of hypophysectomy, 3 weeks after the operation (6-week-old animals), was reduction in the total amount of testicular lipid with a pronounced change in its composition, namely, an increase in the percentage of neutral lipids and a corresponding decrease in the percentage of phospholipids. As the animals became older, this trend was reversed. There was a decrease in the percentage of neutral lipid and a relative increase in the percentage of phospholipids. Changes also occurred in fatty acid composition of the organs of the hypophysectomized animals. In general, these changes consisted of an increase in the percentage of linoleic acid and corresponding lower percentages of arachidonic ($C_{20:4}$) and docosapentaenoic acids ($C_{22:5}$), indicating impairment in the interconversion of unsaturated fatty acids.

Hypophysectomy has a profound influence on the composition of testicular lipids (1–3). Gambal and Ackerman (1) showed that changes occurred in the composition of phospholipids, and that the concentration of the nonphospholipid fraction gradually increased after hypophysectomy in the rat. The studies of Nakamura et al. (3) showed further that the increase in the percentage of neutral lipids was due to an elevation of the percentages of triglycerides (TG), cholesteryl esters (CE) and glyceryl ether diesters (GEDE) at the expense of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The work of Jensen et al. (2) indicated that hypophysectomy impaired, but did not prevent, the conversion of linoleic acid to higher polyunsaturated fatty acids in mature rats with an EFA deficiency. Studies by Goswami and Williams (4) also indicated that the conversion of linoleic acid to other polyunsaturated fatty acids in testicular tissue was impaired by hypophysectomy. In the present investigation the effect of hypophysectomy on lipid metabolism was studied in immature rats by comparison of changes in the composition of the lipid classes and fatty acids with that of normal animals from 6 to 47 weeks of age.

METHODS

Normal and hypophysectomized (1 day after weaning) 3-week-old weanling male rats of the Sprague-Dawley strain were used.[3] The animals were housed in individual cages and fed ad libitum a semipurified diet consisting of 24.5% vitamin-test casein, 53.4% sucrose, 3.7% cellulose,[4]

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[2] Present address: The Danish Fat Research Institute, Lyngby, Denmark.

[3] Obtained from the Hormone Assay Laboratories, Chicago, Ill.

[4] Nonnutritive cellulose, Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

3.7% salt mixture,[5] 1.7% vitamin mix[6] and 13.0% safflower seed oil. Drinking water containing 5% glucose for the first 6 weeks and 5% sucrose thereafter also was supplied ad libitum. The rats were weighed weekly, and at periodic intervals over a period of 41 weeks beginning 3 weeks after the operation (when the rats were 6 weeks of age), groups of 6 animals were killed by exsanguination under an ether anesthesia. The testes, livers, hearts, kidneys and epididymal fat pads were excised, frozen on dry ice, weighed and then stored at −20°. The organs of each group were pooled for lipid analysis which was carried out as previously described (2, 3).

RESULTS

At 6 weeks of age the average daily food consumption of the normal and hypophysectomized (3 weeks after the operation) animals was 10 and 6 g per day, respectively. At 28 weeks of age it was 17 g per day for the normal animals, and 7 g per day for the hypophysectomized animals. Hypophysectomy impaired growth. At 28 weeks of age the mean ± the standard error for the weights of the normal animals was 480 ± 15 g compared to 125 ± 10 for that of the hypophysectomized animals. The organs of both groups of animals consisted of approximately the same percentage of the body weight except for the testes as illustrated by representative results in table 1. The testes of the hypophysectomized animals consisted of a much smaller portion of the body weight (table 1) and were only about one-tenth the size of those of the normal animals as shown in figure 1. The organs of the hypophysectomized animals had generally higher percentages of lipids, especially the testes in accordance with previous observations (1, 3). The relative amounts of neutral and phospholipids in testicular tissue of the normal animals remained fairly constant throughout the experiment at approximately 4 and 14 mg per gram of tissue, respectively. In hypophysectomized animals at 6 weeks of age the neutral lipid was 19.1 mg per gram of testicular tissue and it steadily decreased to 6.5 mg per gram after 18 weeks. The phospholipids of the testes of these animals increased during this period from 12.7 mg to 21.8 mg per gram of tissue.

Most of the hypophysectomized animals in the latter half of the experiment (from

[5] Wesson modified, Osborne-Mendel salt mix (19), from General Biochemicals, Chagrin Falls, Ohio 44022.

[6] The percentage composition of the vitamin mix was: vitamin A acetate (crystals), 0.025; vitamin D_2 conc (440,000 USP units/gram), 0.0175; α-tocopherol, 1.70; *i*-inositol, 0.96; choline chloride, 5.0; menadione, 0.085; p-aminobenzoic acid, 0.325; niacin, 0.43; riboflavin, 0.13; pyridoxine·HCl, 0.035; thiamine·HCl, 0.13; Ca pantothenate, 0.43; biotin, 0.001; folic acid, 0.045; vitamin B_{12}, 0.0002; and casein diluent, 90.77.

TABLE 1

Lipid content and percent body weight of selected organs from normal and hypophysectomized [1] rats

Age	Livers	Hearts	Kidneys	Testes
weeks				
		Normal, % body wt [2]		
6	3.12 ± 0.17	0.34 ± 0.02	0.61 ± 0.02	1.08 ± 0.05
47	2.62 ± 0.20	0.23 ± 0.03	0.51 ± 0.03	0.66 ± 0.04
		Hypophysectomized, % body wt [3]		
6	3.51 ± 0.14	0.36 ± 0.02	0.58 ± 0.04	0.15 ± 0.02
47	2.54 ± 0.21	0.25 ± 0.02	0.37 ± 0.03	0.14 ± 0.02
		Normal, lipid, % wt [3]		
6	5.1	3.0	3.7	1.7
23	4.8	2.7	5.7	1.8
		Hypophysectomized, lipid, % wt [3]		
6	4.6	2.8	4.1	3.2
47	5.6	4.2	6.0	3.6

[1] Operation at age 3 weeks.
[2] Mean ± SE.
[3] Average of pooled samples from six animals.

about week 18) exhibited coarse hair, and scaly tails and feet of varying degrees of severity, characteristic of an EFA deficiency. No attempt was made to evaluate these dermal symptoms because they were unexpected, inasmuch as they had not been encountered in previous work with adult animals (2, 3). A typical example of a severe condition is shown in figure 2.

The course of changes in the lipid classes of the testes during the experiment is indicated in table 2. In accordance with previous studies with adult animals, there was an immediate increase in the percentage of neutral lipid, particularly TG, at the expense of PC and PE in the testicular lipids of the hypophysectomized animals. As the animals became older, this trend reversed itself and, after about week 9, the percentage of phospholipids was greater than the percentage of neutral lipids.

Changes occurred also in the fatty acid composition of the lipids of the organs of the hypophysectomized animals, as illustrated by the results in tables 3 and 4. There was a general increase in the per-

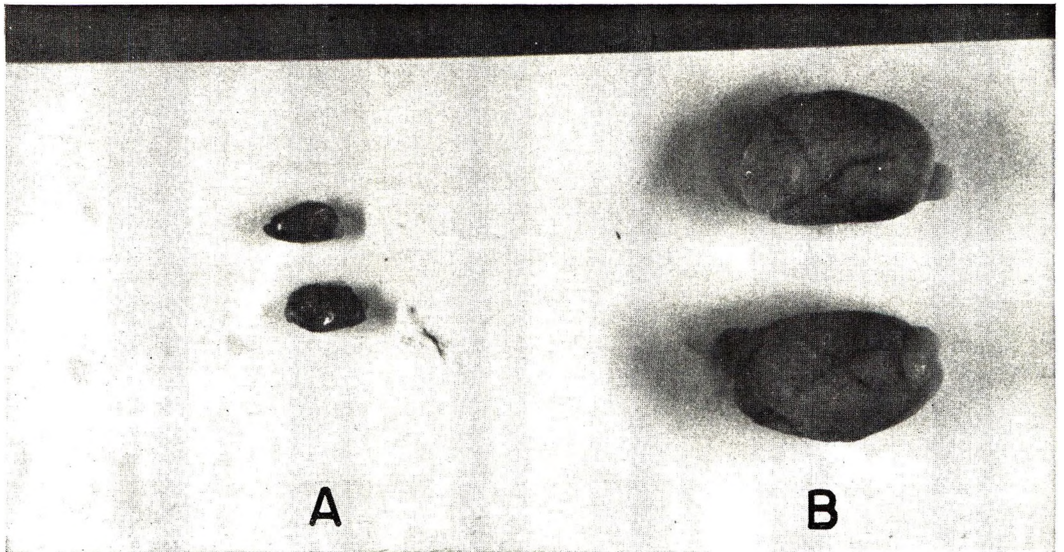

Fig. 1 Comparative sizes of the testes of hypophysectomized (A) and normal (B) animals at 28 and 23 weeks of age, respectively.

TABLE 2

Testicular lipid analysis of normal and hypophysectomized[1] rats

Age	CE[2]	GEDE	TG	FA	C	NL	PL	SU	PE	PC	PS	PI	SPH
weeks						% *wt*							
						Normal							
6	0.6	0.8	5.0	0.5	7.7	15.5	84.5	5.3	23.0	34.5	1.9	10.4	6.0
13	1.2	0.7	10.8	1.4	8.9	23.8	76.2	2.6	23.4	28.0	1.3	10.8	6.7
23	1.2	0.7	11.8	2.5	8.0	24.7	75.3	3.5	22.1	24.6	5.2	11.2	7.0
						Hypophysectomized							
6	3.7	3.3	34.1	6.0	8.8	61.2	38.8	1.9	7.2	12.8	3.0	0.6	5.1
28	1.0	0.7	10.4	4.2	10.0	29.0	71.0	3.8	21.4	26.3	3.3	4.9	7.5
47	1.4	1.1	11.7	1.7	9.6	27.4	72.6	3.7	23.0	27.8	4.1	3.8	8.0

[1] Operation at age 3 weeks.
[2] CE = cholesteryl esters; GEDE = glycerol ether diesters; TG = triglycerides; FA = free fatty acids; C = cholesterol; NL = total neutral lipids; PL = total polar lipids determined by difference, SU = sulfatide; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PS = phosphatidylserine; PI = phosphatidylinositol; SPH = sphingomyelin. Minor and several unknown components in trace amounts not listed.

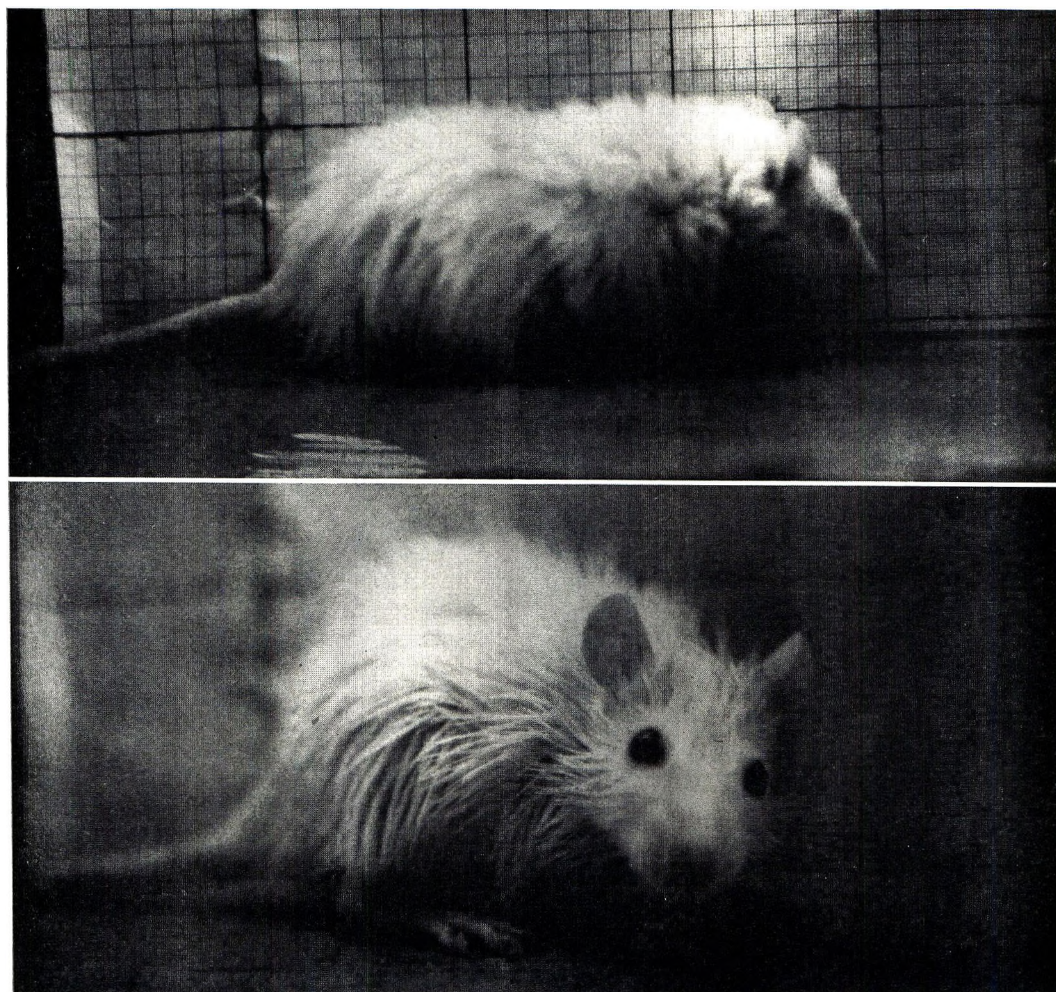

Fig. 2 Hypophysectomized immature rat of the Sprague-Dawley strain at 28 weeks of age.

TABLE 3

Major fatty acids of total lipids of 9-week-old normal (N) and hypophysectomized [1] (HY) rats [2]

	Livers		Kidneys		Hearts		Epididymal fat	
	N	HY	N	HY	N	HY	N	HY
					% wt			
$C_{16:0}$ [3]	18.3	16.6	19.4	14.1	12.7	15.0	19.2	12.4
$C_{16:1}$	2.3	1.2	1.9	1.6	2.0	3.8	4.9	3.1
$C_{18:0}$	13.7	13.7	15.2	9.7	16.4	9.3	3.0	2.5
$C_{18:1}$	10.9	9.5	11.4	11.5	11.6	15.1	20.3	16.6
$C_{18:2}$	26.9	36.3	17.2	43.0	24.8	42.2	49.1	62.2
$C_{20:4}$	18.2	14.7	23.2	11.9	20.7	8.4	0.5	0.3
$C_{22:4}$					2.1	0.4		
$C_{22:5}$					4.7	0.2		

[1] Operation at age 3 weeks.
[2] Average values of pooled samples from six animals.
[3] Shorthand designation for fatty acids; number before colon is chain length, number after colon is number of double bonds.

TABLE 4

Major fatty acids of testicular lipids of 9-week-old normal (N) and hypophysectomized [1] (HY) rats [2]

	Total			PC [3]		PE		TG	
	N	HY	HY [4]	N	HY	N	HY	N	HY
						% *wt*			
$C_{16:0}$ [5]	30.4	22.2	19.3	33.8	36.8	21.9	17.5	25.8	24.8
$C_{16:1}$	1.3	3.0	1.3	1.0	1.5	0.6	—	1.4	1.4
$C_{18:0}$	6.5	8.4	8.9	6.2	8.9	8.4	13.6	2.4	3.3
$C_{18:1}$	8.9	16.9	17.1	12.3	19.3	5.4	12.3	9.7	17.2
$C_{18:2}$	5.1	19.9	9.2	5.4	5.2	2.3	4.2	7.8	37.6
$C_{20:4}$	15.7	9.4	16.9	18.6	16.4	25.2	24.1	4.8	0.9
$C_{22:5}$	19.4	6.1	12.9	16.8	5.6	31.7	21.0	27.1	3.0

[1] Operation at age 3 weeks.
[2] Average values of pooled samples from six animals.
[3] PC = Phosphatidylcholine, PE = phosphatidylethanolamine, TG = triglyceride.
[4] Eighteen-week-old animals.
[5] Shorthand designation for fatty acids; number before colon is chain length, number after colon is number of double bonds.

centage of linoleic acid and a decrease in the percentage of fatty acids that normally arises from their conversion to higher polyunsaturated fatty acids, namely arachidonic ($C_{20:4}$) and docosapentaenoic ($C_{22:5}$).

Fatty acid composition of the major lipid classes of the testes (table 4) showed that changes in the total lipid were largely reflections of those that occurred in the TG. Particularly significant is the effect of hypophysectomy on the testicular TG $C_{22:5}$. This acid was 27.1% in the TG of the testicular lipid of the normal animals compared with 3% in the animals of the hypophysectomized group. Similar changes also occurred in the fatty acid composition of PC and PE of the hypophysectomized animals but were much slower, and these compounds contained appreciable percentages of both $C_{20:4}$ and $C_{22:5}$ at the end of the experiment. Because of the relatively large depletion of TG, at the end of the experiment the total fat reflected generally the fatty acid composition of the phospholipids, that is, relatively large percentages of $C_{20:4}$ and $C_{22:5}$. This trend in composition is illustrated by a comparison of the analysis of the total fatty acid composition of the testes of animals at 9 to 18 weeks of age (table 4).

Differences in the fatty acid composition of the major lipid classes of the livers of normal and hypophysectomized animals for 13-week-old animals (table 5) showed that the same trend of differences in the composition of the fatty acids between normal and hypophysectomized animals occurred in the liver as in testes.

DISCUSSION

Differences in the fatty acid and lipid class composition of the normal and hypophysectomized animals demonstrate well the importance of the hypophyseal hormones in the regulation of lipid metabolism in the immature rat. The effects, logically, are more severe in immature than mature rats previously studied (3) because of the generally higher rate of metabolic activity in the rapidly growing condition. For example, it is difficult to produce the dermal symptoms of an EFA deficiency in adult rats by feeding an EFA-deficient diet (5). In the present study, the increase in $C_{18:2}$ and a decrease in $C_{20:4}$ and $C_{22:5}$ in the hypophysectomized animals indicated an impairment in the interconversion of fatty acids in accordance with tracer studies by Goswami and Williams (4). Interconversion of fatty acids, however, was probably not completely inhibited because in a previous study (2) we showed that interconversion of fed linoleic acid occurred in hypophysectomized EFA-deficient rats, especially in the liver.

The severity of the effects of hypophysectomy in the immature rat serves also as an explanation of the pattern of changes in the lipid classes. Because the phopholipids are important components of membranes, functions of which frequently are involved in hormonal regulation in con-

TABLE 5

Major fatty acids of liver lipids of 13-week-old normal (N) and hypophysectomized [1] (HY) rats [2]

	Total		FA [3]		CE		TG		PL		PC		PE	
	N	HY	N	HY	N	HY	N	HY	N	HY	N	HY	N	HY
							% wt							
$C_{16:0}$ [4]	16.2	16.2	19.1	17.5	8.4	5.4	18.8	18.1	15.3	13.2	17.8	15.2	14.1	10.5
$C_{16:1}$	1.4	1.8	2.4	2.5	3.2	3.0	2.5	2.4	0.6	0.9	0.9	0.8	0.6	0.4
$C_{18:0}$	13.5	14.6	5.6	5.1	1.6	1.9	2.2	3.0	21.1	23.5	23.0	25.1	25.2	27.6
$C_{18:1}$	9.6	8.4	11.4	10.1	25.9	25.0	15.7	13.2	5.3	4.4	4.4	3.7	6.1	5.0
$C_{18:2}$	29.1	36.1	41.0	49.6	43.8	55.4	49.1	53.7	15.9	23.2	11.3	21.0	9.7	18.9
$C_{20:3}$	0.7	1.8	0.7	1.4	0.5	1.1	0.8	1.2	0.6	2.6	0.4	2.4	0.3	2.0
$C_{20:4}$	21.1	14.6	13.7	8.4	12.6	5.7	5.0	2.5	31.0	24.7	35.5	27.6	30.8	27.3

[1] Operation at age 3 weeks.
[2] Average values of pooled samples from six animals.
[3] FA = free fatty acid; CE = cholesteryl esters; TG = triglycerides; PL = total polar lipid; PC = phosphatidylcholine; PE = phosphatidylethanolamine.
[4] Shorthand designation for fatty acids; number before colon is chain length, number after colon is number of double bonds.

trast to triglycerides which serve mainly for storage of energy, they may be expected to be the most sensitive to hypophysectomy and are decreased more rapidly at first. Thus, a relative increase in the percentage of neutral lipid, particularly triglyceride, is observed in the early weeks after hypophysectomy (although there also appear to be simultaneous direct effects involving cholesterol and cholesteryl esters). After the immediate effect on the phospholipids, further depletion of these compounds is resisted because of their role in maintaining essential structures and functions; then the triglycerides are utilized more rapidly. The selective depletion of $C_{20:4}$ and $C_{22:5}$ acids from the triglycerides and the much slower change in the fatty acid composition of the phospholipids indicate that the TG are being used to maintain the composition of the latter compounds. This pattern of changes is reflected in the lipid class and total fatty acid composition. In the early period after hypophysectomy, neutral lipids are in greater concentration than the phospholipids, but at the end of the experiment this relationship is reversed. The fatty acid composition of the total lipid reflects this pattern of changes in lipid composition.

Manifestation of the syndrome of essential fatty acid deficiency in the presence of high concentrations of linoleic acid in the tissues could arise as a result of impairment in the interconversion of this acid to arachidonic acid. Unlike an EFA deficiency in normal animals, there is no increase in 5,8,11-eicosatrienoic acid because the same enzyme system responsible for the conversion of linoleic to arachidonic acid is also involved in the conversion of oleic to higher polyunsaturated acid. However, EFA deficiency may also arise as a direct effect of hypophysectomy on the utilization of arachidonic acid. These aspects of the effects of hypophysectomy are currently under investigation.

ACKNOWLEDGMENT

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Relation Between the Lumen-Blood Electrochemical Potential Difference of Calcium, Calcium Absorption and Calcium-Binding Protein in the Intestine of the Fowl [1]

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ABSTRACT The mechanism of calcium absorption by the intestine in vivo was investigated in both chicks and laying hens. Calcium activity, measured in ultrafiltrates of intestinal contents and blood plasma, and the electrical potential (PD) between the intestinal lumen and circulation, were used to calculate the electrochemical potential difference of calcium (ECPD) between lumen and blood. The calcium level of the hens' diets was 3.94, 1.76, or 0.60%; that of the chicks' diets was 1.11, 0.71 or 0.31%. The same diets were also used to measure calcium absorption in laying hens with the aid of yttrium-91, and calcium retention in chicks by bone analysis. Included were also measurements of the calcium-binding protein (CaBP) in the duodenal mucosa. In hens, the ECPD in the calcium-absorbing intestinal segments, the duodenum and jejunum, decreased as dietary calcium decreased but remained always positive. Thus, absorption always proceeded in the direction of the driving forces (downward). The ECPD in the chick upper intestine was positive with the two higher calcium diets, but became negative in the entire intestine with the 0.31% calcium diet. Thus, absorption proceeded downward with the 1.11 and the 0.71% calcium diets, but upward (against the driving forces) with the 0.31% calcium diet. It was concluded that, under conditions of calcium restriction in chicks, active transport may assume an important role in the overall process of calcium absorption. The CaBP was higher in laying hens than in chicks; it was independent of dietary calcium in the hen for the range of intake studied, but in chicks it increased as dietary calcium was reduced below 0.71%. The possible association of the CaBP with the adaptation to low calcium intakes is discussed. A net inflow of calcium was observed in the ileum of the hens fed the 3.94% calcium diet but not with diets containing less calcium. Phosphate absorption decreased progressively with increased dietary calcium supplementation. The duodenum was identified as the major site of this antagonism.

Analysis of the relationship between the driving forces and the transport of a nutrient across the intestinal epithelium may provide valuable information on the mechanism of absorption of this nutrient. Such an analysis, with regard to calcium, was applied to in vitro (1, 2) and in situ (3, 4) systems, but to the best of our knowledge has not yet been applied to the intestine of the intact animal.

In vitro transport frequently correlates poorly with absorption in vivo (5). Therefore, the understanding of the principal mechanisms responsible for transport in the intact animal must ultimately depend on in vivo measurements.

In a previous report (6), comparisons were made among the various intestinal segments with regard to the relationship between the lumen-blood electrochemical potential difference of calcium (ECPD) and its in vivo absorption. In the present study the analysis was extended by modifying the in vivo ECPD through manipulations of the dietary calcium level, and estimation of absorption, under the same conditions.

In recent publications (7–10) Wasserman and associates described a vitamin D-dependent calcium-binding protein (CaBP) in the intestinal mucosa of chicks, laying hens and rats, and suggested that this protein was intimately involved in the trans-

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location of calcium across the intestinal epithelium. The present study provided a good opportunity to examine further the possible association of this protein with the in vivo absorption on the one hand, and the driving forces operating on intestinal calcium, on the other hand.

MATERIALS AND METHODS

Animals. a) Laying hens (White Leghorn hens 8 to 15 months old) were used. They were kept in individual laying cages situated in an open shed, and were fed a commercial diet containing 3.5 to 3.7% calcium. Since we have previously demonstrated changes in calcium absorption during the laying cycle (11), hens were killed only during shell formation. b) Chicks (White Rock males) were used. For measurements of intestinal parameters they were kept until 4 to 5 weeks of age in electrically heated battery brooders and fed a commercial starter diet containing about 1.0 to 1.2% calcium.

For analysis of intestinal content, the birds were killed with an intracardiac injection of sodium pentobarbital. Appropriate intestinal segments (11) were ligated and the entire intestine was then removed. The content of each segment was emptied and taken for analysis.

Diets. The main dietary calcium supplements were calcium carbonate and dicalcium phosphate. The latter was used as the phosphate supplement in the layers' diets (table 1), whereas a mixture of 1:1 monosodium and potassium phosphate supplied the phosphate in the chicks' diets (table 2). Dietary calcium did not exceed the level used in practice, i.e., 4% in laying hens and 1.2% in chicks.

Preparation of ultrafiltrates. The intestinal contents were emptied into Visking tubing, 24/32 inch, and ultrafiltrates were prepared under positive pressure, as described previously (5).

Assay procedures. Calcium was determined by direct EDTA titration with Calcein as indicator under ultraviolet light, or by EDTA titration with Hydroxy-Naphthol blue,[2] following oxalate precipitation and hydrogen peroxide oxidation (13).

Phosphorus was determined by the method described by Gomori (14).

TABLE 1

Composition of the experimental diets for laying hens

Diet no.	1	2	3
	%	%	%
Constant ingredients [1]	33.85	33.85	33.85
Calcium carbonate	8.00	2.60	—
Milo	58.15	63.55	66.15
Calcium content, % [2]	3.94	1.76	0.60
Phosphorus content, % [2]	0.69	0.68	0.70

[1] In percent of diet: Soybean oil meal (45% protein), 20.00; soybean oil, refined, 1.00; wheat bran, 5.00; fish meal, 3.00; alfalfa meal, dehydrated, 3.00; vitamin mixture, 0.25; salt mixture, 0.30; dicalcium phosphate, 1.30. For composition of vitamin and salt mixtures, see Hurwitz and Bornstein (12).

[2] By chemical analysis.

TABLE 2

Composition of the experimental diets for chicks

Diet no.	1	2	3
	%	%	%
Constant ingredients [1]	38.20	38.20	38.20
Calcium carbonate	2.50	1.50	0.50
Milo	59.30	60.30	61.30
Calcium content, % [2]	1.11	0.71	0.31
Phosphorus content, % [2]	0.66	0.66	0.66

[1] In percent of diet: Soybean oil meal (45% protein), 33.50; soybean oil, refined, 2.50; DL-methionine, 0.15; sodium phosphate, monobasic, 0.68; potassium phosphate, monobasic, 0.67; choline chloride (50%), 0.10; vitamin mixture, 0.30; mineral mixture, 0.30 (12). The vitamin mixture supplied: (per kg diet) Vitamin A, 10,000 IU; vitamin D_3, 1,500 ICU; alpha-tocopherol, 5 IU; and: (in mg/kg diet) menadione, 2; thiamine, 25; riboflavin, 16; Ca pantothenate, 20; pyridoxine, 6; biotin, 0.6; folic acid, 4; niacin, 150; vitamin B_{12}, 0.02; ascorbic acid, 200.

[2] Determined by chemical analysis.

Calcium activity was assessed in ultrafiltrates of blood plasma and intestinal contents, with an Orion calcium electrode and a Keithley electrometer 610B, as described previously (6). A recorder connected to the electrometer facilitated the recordings of stable electrode potentials.

For measurements of yttrium-91, samples of acid-dissolved ash were placed in counting vials containing Bray solution, and counted in a TriCarb liquid scintillator.

Measurements of the transmural electrical potential (PD). The PD was measured between two agar-KCl bridges connected through calomel electrodes to a Keithley electrometer and recorder, as described previously (6). One bridge was placed in

[2] Mallinckrodt Chemical Works, St. Louis, Mo.

the intestinal lumen and the other in the brachial vein of anesthetized birds.

Assay of calcium-binding protein (CaBP). Birds were killed by an intracardiac injection of sodium pentobarbital; the duodenum was immediately removed and placed in ice-cold 120 mM sodium chloride solution. The duodenum was then everted and the mucosa was scraped off, homogenized in a standard volume of Tris buffer, and centrifuged at 33,000 × *g*. The supernatant was then analyzed for CaBP with calcium-45 and Chelex-100 as described by Wasserman et al. (8). The results are expressed as percent of the added calcium-45 which remained in solution.

RESULTS

Series 1. The purpose of this series of experiments was to evaluate the dependence on dietary calcium of the electrochemical potential difference of calcium between the intestinal lumen and circulation (ECPD). Groups of 11 laying hens and chicks each, were fed the respective diets detailed in tables 1 and 2. The measurements which were taken between the fifth and the eighth day of feeding included the determination of the calcium activity in blood plasma and intestinal contents, and the assessment of the lumen-blood PD.

Measurements were also made of ultrafilterable and total calcium and phosphate in intestinal contents; results will be given elsewhere.[3]

The activity of calcium in the intestinal contents and the blood-lumen PD are presented in tables 3 and 4, for laying hens and chicks, respectively. The calcium activity and PD for the hens fed the high calcium diet (3.94%) were similar to those reported by us previously (6); the activity increased from the duodenum to the jejunum, but decreased progressively in

[3] Hurwitz, S., and A. Bar 1969 Unpublished results.

TABLE 3

Calcium activity (a_{Ca}) in the intestinal contents and blood-lumen electrical potential difference (PD) in hens fed different levels of dietary calcium (series 1)[1,2]

Intestinal segment	Dietary Ca, %					
	3.94		1.76		0.60	
	a_{Ca}	PD	a_{Ca}	PD	a_{Ca}	PD
	mM	*mv*	*mM*	*mv*	*mM*	*mv*
Duodenum	4.69 ± 1.12 [3]	11.6 ± 3.7	3.79 ± 1.13	10.2 ± 3.4	2.20 ± 0.22	9.8 ± 1.5
Jejunum	6.44 ± 1.71	9.1 ± 4.0	3.66 ± 0.98	8.2 ± 3.7	2.12 ± 0.43	9.0 ± 1.9
Ileum, upper	3.38 ± 0.67	5.4 ± 2.1	1.99 ± 0.57	6.6 ± 3.1	0.94 ± 0.48	5.6 ± 1.8
Ileum, lower	2.13 ± 0.51	12.1 ± 6.9	1.34 ± 0.26	13.8 ± 8.8	0.67 ± 0.19	9.5 ± 2.8

[1] a_{Ca} was measured with a Orion calcium electrode in ultrafiltrates from intestinal contents. The PD was measured between two agar bridges placed in the brachial vein and intestinal lumen, respectively. The blood was always positive with regard to the intestinal lumen.
[2] The activity of calcium in the blood plasma was 0.75, 0.72 and 0.76 mM for the 3.94, 1.76 and 0.60% calcium diets, respectively.
[3] Averages ± SD; n = 6 and n = 5 for a_{Ca} and PD, respectively.

TABLE 4

Calcium activity (a_{Ca}) in the intestinal contents and blood-lumen electrical potential difference (PD) in chicks fed different levels of dietary calcium (series 1)[1,2]

Intestinal segment	Dietary Ca, %					
	1.11		0.71		0.31	
	a_{Ca}	PD	a_{Ca}	PD	a_{Ca}	PD
	mM	*mv*	*mM*	*mv*	*mM*	*mv*
Duodenum	5.87 ± 1.35 [3]	10.2 ± 2.8	4.16 ± 1.05	8.3 ± 2.0	2.19 ± 0.50	14.2 ± 3.8
Jejunum	5.99 ± 1.91	10.3 ± 2.9	4.92 ± 1.56	10.5 ± 4.3	1.78 ± 0.33	15.5 ± 2.6
Ileum, upper	5.39 ± 1.69	11.6 ± 2.1	3.18 ± 0.58	9.1 ± 3.6	1.25 ± 0.10	10.2 ± 2.5
Ileum, lower	2.75 ± 1.01	14.8 ± 6.9	2.11 ± 0.32	15.5 ± 8.5	0.70 ± 0.11	15.2 ± 4.7

[1] See footnote 1, table 3.
[2] The activity of calcium in the blood plasma was 0.91, 0.89 and 0.86 mM, for the 1.11, 0.71 and 0.31% calcium diets, respectively.
[3] Averages ± SD; n = 6 and n = 5 for a_{Ca} and PD, respectively.

the upper and the lower ileum. The same pattern was observed also for the lower calcium diets with the exception that here the activity was similar in the duodenum and jejunum. At all levels of the intestine, calcium activity was positively related to dietary calcium.

The PD in laying hens (table 3) was similar to that reported previously (6), with no significant differences between the dietary treatments.

Although the diets of the chicks contained considerably less calcium than those of laying hens, the calcium activity in the intestinal contents was maintained at similar levels (table 4). Thus, less calcium is required in the diet of the chick than that of the hen to maintain the same calcium activity.

The changes in calcium activity along the intestine of the chick were similar to those in laying hens, except that the decrease from the jejunum to the upper ileum was lower in magnitude. In the chick intestine, the calcium activity again was positively related to dietary calcium.

The PD in the chick intestine was, in general, similar to that in laying hens. It was, however, somewhat higher in the duodenum and jejunum of the chicks fed the 0.31%-calcium diet as compared to the two higher calcium diets.

Series 2. In this series, calcium absorption as a function of dietary calcium was evaluated in laying hens.

Thirty hens were placed in metabolism cages and fed the high calcium diet (diet 1, table 1) for a two-week period of adjustment. Groups of eight hens each were then fed the experimental diets (table 1) which were labeled with yttrium-91 at a concentration of about 5 μCi/kg. The method of incorporation of the isotope into the diet and the system of analysis were described previously (11).

After 4 days on the respective experimental diets the hens were killed during shell secretion and their intestines were separated into the appropriate segments. The contents of each segment were transferred into plastic beakers and kept frozen until analyzed. Feed intake was recorded for the last 2 days of the experiment.

All samples were analyzed for calcium, phosphorus and yttrium-91. For data presentation, the ratio of mineral to yttrium-91 in the respective segment was divided by the ratio in the diet and multiplied by 100. The cumulative percentage of net absorption down to any level of the intestine, can be readily obtained by subtracting the value read from the graph (fig. 1) from 100.

The change in the calcium/yttrium-91 ratio along the intestine (fig. 1, lower graph) for the hens fed the 3.94% calcium diet had a pattern similar to that observed previously (11) for hens during shell formation. With all dietary treatments, most of the calcium was absorbed in the proximal segments, the duodenum and jejunum. The fraction of the dietary calcium absorbed increased in those segments with

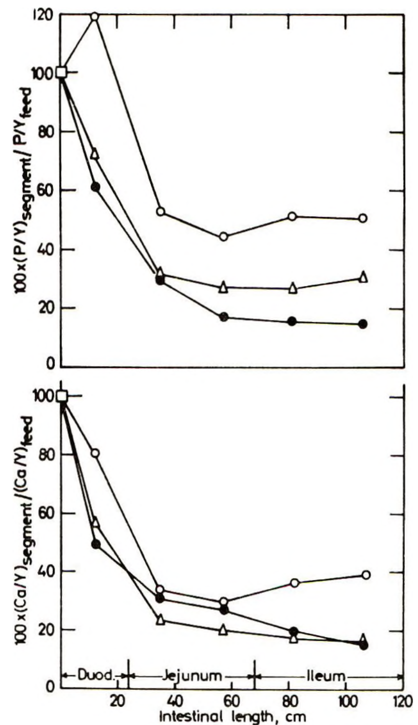

Fig. 1 Mineral/yttrium-91 ratios in the intestine of laying hens fed diets containing 3.94% (○), 1.76% (△) and 0.60% (●) calcium, respectively. Upper graph, phosphorus/yttrium-91 ratio; lower graph, calcium/yttrium-91 ratio. All values were standardized to a dietary mineral/yttrium-91 ratio of 100 (for explanation, see text, Series 2).

the lowering of the dietary calcium level, but the differences among the diets were not very large.

An increase in the calcium/yttrium-91 ratio in the ileum of the birds fed the 3.94% calcium diet signifies a net inflow of calcium into this segment. Such an inflow was also noted previously with birds fed a similar diet (11). Hardly any change in calcium/yttrium-91 took place in the ileum of the birds fed the 1.74% calcium diet, whereas with the low calcium diet (0.60%) the ratio decreased slightly, indicating some net outflow of calcium.

Phosphate absorption (fig. 1, upper graph) was practically complete at the lower jejunum, similar to that of calcium. A net inflow of phosphate occurred in the duodenum of the birds fed the 3.94% calcium diet, whereas a net outflow was observed with the diets containing less calcium. A net inflow occurred also in the ileum but only with the high calcium diet; this inflow was, however, much lower in magnitude than that which had occurred in the duodenum. As a result mainly of dietary effects in the duodenum, the overall absorption of phosphate was adversely affected by increasing levels of dietary calcium.

Series 3. Due to technical difficulties in applying our method of reference substance in young chicks, the retention of calcium in the skeleton, rather than intestinal absorption, was evaluated.

The tibia was selected as an index of calcium retention because it has been shown that the ratio of tibia- to total body-calcium is quite constant in young chicks fed diets of different calcium content (15).

Ten-day-old chicks were assigned to four groups according to their body weights in order to ensure the same weight distributions in each group. Chicks of one group were killed immediately and their tibias were taken for analysis. The other 3 groups were fed the experimental diets (table 2) for 2 weeks. At the end of the this period the chicks were killed and their tibias were retained.

The gain in tibia calcium was taken as the difference between the calcium content of the tibias of the experimental groups and that of the group killed at the start of the experiment. The calculated gains in tibia calcium were 136, 90 and 46 mg for the 1.11, 0.71 and 0.31% calcium diets, respectively. Thus, significant amounts of calcium were retained even with the most calcium-deficient lot.

Series 4. The CaBP was measured in the duodenal mucosa of laying hens and chicks which had been fed the diets detailed in tables 1 and 2, respectively, for a period of 5 days.

In agreement with Wasserman et al. (8), the CaBP level was higher in laying hens than in chicks (table 5). Dietary calcium did not influence the CaBP significantly ($P > 0.05$) in laying hens, but in chicks the CaBP increased as dietary calcium decreased. The relationship between the CaBP and dietary calcium in chicks does not appear to be linear.

DISCUSSION

With existing techniques such as used in this study, it is impossible to obtain instantaneous measurements of intestinal calcium activity, due to difficulties in obtaining reliable potentials in suspensions and to the slow equilibration of the calcium electrode. The calculations of the electrochemical potential difference (ECPD) are made with the assumption that the activity of calcium does not change appreciably between sampling and measurement.

The ECPD was calculated from the equation

$$\frac{(\tilde{\mu}_l-\tilde{\mu}_b)}{F} = \frac{RT}{F}\ln\frac{a_l}{a_b} + Z(\psi_l-\psi_b),$$

where $(\tilde{\mu}_l-\tilde{\mu}_b)$ is the ECPD between the in-

TABLE 5

Assay of the calcium-binding protein level (CaBP) in the duodenal mucosa of chicks and laying hens fed diets different in calcium content (series 4) [1]

Dietary Ca	CaBP
%	% dose
Laying hens	
3.94	32.5 ± 1.7 [2]
1.76	38.1 ± 4.9
0.60	39.3 ± 3.1
Chicks	
1.11	12.3 ± 1.0
0.71	15.1 ± 1.8
0.31	21.8 ± 1.0

[1] Assayed according to Wasserman et al. (8). For explanation of units, see text.
[2] Average from six birds ± SEM.

testinal lumen and blood plasma; a_l and a_b are the activities of calcium in the lumen and blood plasma, respectively; $(\psi_l-\psi_b)$ is the lumen-blood PD; F, R, T, and Z are the Faraday constant, gas constant, absolute temperature and valency, respectively.

The amount of calcium absorbed by any intestinal segment was calculated from the change in the relative calcium/yttrium-91 ratio (fig. 1) multiplied by the average hourly calcium intake.

The ECPD in the laying-hen intestine changed with changes in the dietary calcium level, but remained always positive in the duodenum and jejunum where most of the absorption occurred (table 6). Thus, with dietary calcium levels ranging between 0.60 and 3.94%, calcium was always transported downward. In these anterior segments, there was no indication of any saturation of the absorptive mechanism as the ECPD increased. This could be taken as evidence that calcium transport in the laying-hen intestine is not carrier-mediated, although the same result could be obtained with a carrier-mediated transport system if the carrier were far from saturation (16). Direction of calcium flow and the apparent lack of saturation kinetics are both consistent with simple diffusion as the principal mechanism of calcium transport in the hen.

The pattern of changes in the ECPD along the intestine of the chick (table 7) was similar to that observed for laying-hen intestine. With the two higher calcium diets, the ECPD was positive in all intestinal segments except for the lower ileum. With the lowest level of dietary calcium however, the ECPD was slightly negative in the duodenum and became decidedly negative in the jejunum and ileum. Since calcium absorption occurred with all diets, it appears that calcium was transported downward with the two higher calcium diets, and upward with the lowest calcium diet. Unless the presence of driving forces other than the ECPD is evoked, calcium appears to be transported actively with the low level of dietary calcium, according to Rosenberg's criterion (17).

Wasserman and Kallfelz (3), following studies with the chick intestine in situ, suggested a change in the principal mechanism of absorption as the luminal concentration of calcium was increased. Furthermore, active transport of calcium by the rat intestine in vitro could be demonstrated only with calcium concentrations lower than 2 mM (2). It thus seems possible that when the ECPD is low, calcium would be transported by an active mechanism, but at higher ECPD's mainly by diffusion.

The increase in the ability of the intestine to transport calcium actively, in response to a restricted calcium intake (18) could provide one of the means of adaptation to such nutriture as described for various animals (19). Assuming that CaBP plays an important role in calcium transport, as suggested by Wasserman, the present results point to the appearance of CaBP as a possible mechanism of adaptation to low calcium intake in the chick. In this animal, CaBP hardly changed while

TABLE 6

Relationship between the electrochemical potential difference of calcium and the intestinal calcium absorption in laying hens

Intestinal segment	Dietary Ca, %					
	3.94		1.76		0.60	
	ECPD [1]	J_{net} [2]	ECPD	J_{net}	ECPD	J_{net}
	mv	*mmoles/hr*	*mv*	*mmoles/hr*	*mv*	*mmoles/hr*
Duodenum	26.4	1950	23.6	1080	9.3	320
Jejunum	39.8	815	27.5	321	8.3	57
Ileum, upper	32.8	−199	13.3	18	−5.6	26
Ileum, lower	3.9	−40	−10.9	−52	−22.4	0

[1] ECPD denotes the electrochemical potential calcium difference of calcium between the intestinal lumen and blood calculated from the luminar and plasma calcium activities and lumen-blood PD (opposite in sign to blood-lumen PD), given in table 3.

[2] J_{net} denotes the net transport of calcium out of the intestine, calculated from the change in the ratio of calcium to yttrium-91 (fig. 1). A negative value signifies a net inflow into the intestinal segment.

TABLE 7

Electrochemical potential difference of calcium (ECPD) across the intestine of chicks fed diets of different calcium content [1]

Dietary Ca, %	1.11	0.71	0.31
	mv	*mv*	*mv*
Duodenum	28.3	20.9	−3.2
Jejunum	30.2	25.1	−11.2
Ileum, upper	24.8	16.1	−10.4
Ileum, lower	−0.3	−7.7	−28.6

[1] See footnote 1, table 6. The ECPD was calculated from the results given in table 4.

the ECPD remained positive, but was markedly elevated when the ECPD became negative. An increase in CaBP induced by changes in dietary calcium level was not observed in this experiment, with laying hens in which the ECPD remained always positive in the calcium absorbing segments.

In the upper ileum of the hens which had received the high calcium diet, a net inflow of calcium occurred against an ECPD favorable for transport in the opposite direction. By definition (17), that would mean an active transport of calcium into the ileal lumen. Although such active transport is consistent with a model proposed by Holdsworth (20), it is still difficult to accept. An alternate explanation can be based on the assumption of more than a single pathway for calcium movement into the intestinal lumen, due to the heterogeneous nature of the intestinal epithelium. One such pathway may be diffusional and depend on the intraluminal calcium activity (2, 21). Another, which includes digestive juice calcium, could depend on the calcium concentration in body fluids. With a limited permeability of the ileum to calcium, the sum of the various inflow pathways may exceed the outflow, resulting in a net inflow of calcium.

In agreement with previous observations (13), phosphate absorption occurred mostly in the jejunum. Also in agreement with the same results and with those of Shirley et al. (22) is the occurrence of a net inflow of phosphate in the duodenum of the birds fed the high calcium diet. Such an inflow was not observed, in the present study, with birds fed 1.76% calcium or lower. The net inflow of phosphate probably resulted from an inhibition of phosphate outflow in the presence of high calcium concentrations due to massive coprecipitation of calcium and phosphate in the duodenum.[4]

A net inflow of phosphate had also occurred in the ileum of the high-calcium birds. The magnitude of this inflow, however, was small compared with that observed in the duodenum.

The inhibitory effect of increasing levels of dietary calcium on the absorption of phosphate is well recognized (23). The present results identify the duodenum as the main site of this inhibition in the laying hen.

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[4] See footnote 3.

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Sodium Metabolism and Its Requirement during Reproduction in Female Rats [1,2]

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ABSTRACT Sodium metabolism and reproductive performance were studied in 80 young female rats fed a corn-soybean meal-casein diet containing sodium at one of five levels from 0.01 to 0.09%. Data were obtained on a) the age and body weight when vaginal opening occurred; b) length of estrus cycle and duration of estrus; c) efficiency of mating; d) feed intake and body weight changes during gestation; e) litter size and pup birth weight; f) water, fat and sodium content of newly born pups; g) changes in hematocrit and plasma sodium during gestation; h) bone sodium content, and i) histology of the adrenals and kidneys. The rats fed 0.01% sodium gave birth to some viable pups but practically all measures of reproductive performance were adversely affected. With 0.03% dietary sodium, litter size was not affected but the pups were smaller at birth. No differences in reproduction or tissue sodium were found among the rats fed either 0.05, 0.07 or 0.09% sodium although a difference was noted in the juxtaglomerular indices.

Estimates of the sodium requirements of rats during growth and during gestation are given as 0.05 and 0.5% of the diet by the National Research Council (1). The estimate of the growth requirement, based on the study of Grunert et al. (2), is well established whereas the estimate for gestation, obtained from the work of Miller (3) and of Olson and St. John (4) is not so well founded and appears unreasonably large. No physiological basis for such a large difference in sodium requirements is known. In fact, Kirksey and Pike (5) state that a diet containing 0.14% sodium has been used routinely for pregnancy studies with rats. These authors were unable to demonstrate any effect on reproductive performance of a diet containing 0.025% sodium but noted adverse changes in the maternal animals.

The present study was designed to quantitate more accurately the sodium requirement of rats during gestation and also to obtain further information regarding the sodium metabolism of pregnant rats.

EXPERIMENTAL PROCEDURES

Eighty young female albino rats from a strain maintained at this laboratory were selected for similar body weight in groups of five during a 4-day period as they attained approximately 50 g body weight. The rats in each group were assigned at random to five dietary treatments which consisted of a basal diet supplemented with sodium chloride to provide 0.01, 0.03, 0.05, 0.07 or 0.09% sodium. The composition of the basal diet is given in table 1. All animals were housed individually in galvanized cages with wire mesh bottoms. Environmental temperature was maintained close to 22° in a well-ventilated room. Feed and demineralized water were supplied ad libitum.

At the start of the experiment each animal was examined daily and its age and body weight at the time of opening of the vaginal orifice was recorded.

Starting at about 15 weeks of age, the estrus cycle patterns were followed by microscopic examination of vaginal smears taken daily, or every 12 hours in certain stages of the cycle. The duration of estrus and the total length of the estrus cycles were calculated from these observations. When proestrus was detected for the fourth time, the females were placed with a male for mating. All the females in each block were mated with the same male. Matings

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TABLE 1
Composition of basal diet [1]

	%
Corn	71.0
Soybean meal	12.0
Casein	10.0
Sucrose and vitamin mix [2]	4.0
Sodium free salt mixture [3]	3.0

[1] Supplied with additional NaCl to provide 0.01, 0.03, 0.05, 0.07 and 0.09% sodium in the diet. A number of analyses of these diets showed the following percentages of sodium: 0.009 ± 0.001, 0.029 ± 0.004, 0.050 ± 0.004, 0.070 ± 0.003 and 0.089 ± 0.004 on the air dry basis. The diet contained 0.45% potassium by analysis.

[2] Supplied the following per kg diet: (in IU) vitamin A, 4000; vitamin D, 6000; (in mg) *dl*-α-tocopherol acetate, 120; menadione, 0.2; thiamine·HCl, 2.5; riboflavin, 8.0; niacin, 30.0; calcium pantothenate, 16.0; pyridoxine·HCl, 2.4; biotin, 1.0; folic acid, 1.0; and vitamin B_{12}, 10.0 μg.

[3] Mineral premix: $CaHPO_4$, 96.333%; $MnSO_4 \cdot H_2O$, 0.620%; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.303%; $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, 0.093%; and KI, 0.001%.

were confirmed when sperm cells were detected in the vaginal smears. In order to observe any failure to conceive and to indicate whether any interruption of pregnancy occurred, the vaginal smears were continued after mating until vaginal bleeding was noticed, usually on day 10 to day 12 of pregnancy.

Individual body weights and feed consumptions were recorded every third day during the 3-week gestation period. On day 21 of gestation each animal was moved to a separate cage with wood shavings (contained 0.0027 to 0.0035% Na) for bedding. The animals were checked several times daily for the occurrence of parturition and the number and birth weight of pups was recorded as soon as they were noted after parturition was completed.

Blood samples of about 0.1 ml were taken in duplicate from the tail vein in ammonium heparinized capillary tubes on the day of mating and on day 21 of pregnancy for determination of hematocrits and plasma sodium concentrations.

After parturition, five or more rats from each dietary group were killed with ether for organ and tissue examinations. The carcasses of the rats from all except the lowest dietary sodium group were stored in the freezer for a period before removal of the bones for sodium analyses. Bones were removed immediately after killing from the animals of the lowest sodium group. Both femurs from each rat were removed, scraped free of periosteum, the epiphyses cut off and the shafts blown and scraped free of marrow before being weighed and dried to constant weight at 100°. The dried bones were digested with a small quantity of nitric acid and diluted with water for sodium analysis.

A total of 12 pups were removed from each experimental group soon after parturition and analyzed for water, fat and sodium. The pup carcasses were individually weighed, placed in ashless filter paper thimbles and lyophilized. The lyophilization prevented the loss of body fluids with attendant electrolytes which had previously been found to occur in oven drying. Dryness was subsequently checked by oven drying at 100°. The dried carcasses were extracted with ether in a large Soxhlet extractor and fat was calculated as the weight loss during extraction. The fat-free dry carcasses were ashed in a muffle furnace at 550°, the ash was taken up in dilute HCl and made to volume with water for sodium analyses. Plasma sodium determinations were made with a flame photometer, and bone and carcass sodium determinations were made with an atomic absorption spectrophotometer. The standards were cross checked with both instruments.

The kidneys and adrenals were removed immediately from the animals that were sacrificed after parturition. Kidney sections 4 μ thick were taken at right angles to the longitudinal axis at the midpoint and stained by the method of Pitcock and Hartroft (6). Three sections from each animal were scored for juxtaglomerular granulation according to the method of Hartroft and Hartroft (7), in which the degree of granulation is expressed as a juxtaglomerular index. The adrenal sections, also 4 μ thick and taken similarly to those from the kidney, were stained with hematoxylin and eosin, and the total area of adrenal zona glomerulosa from each of three sections for each animal was determined. The sections were projected on a screen at a known magnification using a Leitz-Prado micro-projector, the zona glomerulosa areas were traced on transparent paper, measured with a planimeter and the true areas calculated. The total number of cells in the area was counted by using a square grid of known area in the microscope and the count was made at two locations in each of three sec-

tions for each animal. The average area of a cell and the total number of cells in in the cross sectional area of the zona glomerulosa were calculated.

Differences between treatment means were assessed with Duncan's (8) new multiple range test.

RESULTS AND DISCUSSION

Vaginal opening is commonly used as an indicator of sexual maturation in rats. No difference was found in body weights at the time of vaginal opening, but the age of the rats fed the 0.01% sodium diet was significantly greater (table 2). Orent-Keiles et al. (9) also noted delayed sexual maturity in rats fed a low (0.002%) sodium diet. Much variability occurs in the mean age and body weight at the time of vaginal opening reported in the literature as summarized by Mandl and Zuckerman (10). Engle et al. (11) suggested that vaginal opening is more closely correlated with body weight and length than with age whereas Bogart et al. (12) found a smaller coefficient of variation for age (4.9%) than for body weight (10.6%) at the time of vaginal opening. Asdell and Crowell (13) noted an increased age as well as a decreased body weight at the time of vaginal opening, when growth was restricted. Restriction of growth caused by reduced dietary sodium in the present experiment led to an increased age but not decreased body weight at the time of vaginal opening.

A delay in the commencement of estrus activity was found in the rats fed the lowest sodium diet. Regular cycles were occurring in all rats except the 0.01% sodium group at the time the observations were started. The data for the average length of the cycle and duration of estrus in the different dietary groups are given in table 3. Some rats in the lowest sodium group were mated before the observation of three cycles was completed and the data for the third cycle in this group are based on fewer observations. No statistical treatment of the data was made, but the rats fed 0.01% dietary sodium showed distinctly longer estrus cycles, a greater duration of estrus and more variation among rats within the group.

The number of matings required for conception, given in table 4, was calculated both as the number of times the females were placed with a male and also as the number of times sperm was found in the vaginal smears. Again, the only difference observed was in the rats fed the 0.01% sodium diet. These rats required approximately twice as many matings for conception (calculated in either fashion) as the rats of other groups. Female rats fed a very low sodium diet (0.002%) by Orent-Keiles et al. (9) were not observed to copulate when placed with males fed a stock ration.

Body weights at the start and end of gestation and after parturition, and feed intake during gestation are shown in table

TABLE 2

Age and body weight of rats at time of vaginal opening

Dietary Na	No. of rats	Age [1,2]	Body weight [1]
%		*days*	*g*
0.01	16	57.2 ± 5.05 [a]	104.6 ± 7.3
0.03	22	36.7 ± 0.93 [b]	98.6 ± 4.5
0.05	22	34.6 ± 0.67 [b]	101.5 ± 3.6
0.07	22	34.6 ± 0.74 [b]	101.4 ± 3.1
0.09	16	35.8 ± 0.58 [b]	104.7 ± 2.6

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

TABLE 3

Length of estrous cycle and duration of estrus

Dietary Na	Estrous cycle [1,2]			Mean [1,2]
	1	2	3	
%	*hr*	*hr*	*hr*	*hr*
0.01	275.8(42.2)	239.8(47.2)	259.3(54.7)	258.3(48.0)
0.03	121.5(34.8)	122.8(36.8)	118.0(33.3)	120.8(35.0)
0.05	121.3(36.6)	125.8(39.7)	128.5(41.7)	125.2(38.2)
0.07	127.6(38.6)	122.1(37.9)	126.0(38.0)	125.2(38.2)
0.09	121.6(38.8)	124.0(36.8)	120.3(34.5)	122.0(36.7)

[1] Sixteen rats per group.
[2] Figures outside parentheses are length of estrous cycle with duration of estrus inside parentheses.

TABLE 4

Number of services required for conception

Dietary Na	No. of services required [1,2,3]	
%		
0.01	3.31 ± 0.25 [a]	(2.12 ± 0.24) [a]
0.03	1.59 ± 0.23 [b]	(1.11 ± 0.08) [b]
0.05	1.67 ± 0.18 [b]	(1.11 ± 0.08) [b]
0.07	1.28 ± 0.13 [b]	(1.06 ± 0.06) [b]
0.09	1.59 ± 0.21 [b]	(1.06 ± 0.06) [b]

[1] Mean ± SEM; 16 rats per group.
[2] The first figure indicates the number of times the females were put with males for mating; the figures in parentheses are numbers of matings confirmed by the detection of sperm in the vaginal smear.
[3] Values with unlike superscripts are significantly different, $P < 0.01$.

5. Maternal body weights were reduced slightly during gestation in the rats fed 0.01% sodium, increased slightly in the rats fed 0.03% sodium and increased moderately in rats fed the three higher sodium diets. The weight gains of the rats fed 0.05% or more sodium were similar to those reported by Murray (14) for adequately fed rats during their first pregnancy, but somewhat less than the maternal gains with unrestricted feed intake recorded by Chow and Lee (15). Hammond (16) states that tissues of high metabolic activity, such as placenta, fetus or brain can draw on maternal tissues of lower metabolic activity when a nutrient shortage occurs. In this experiment, where sodium was the limiting nutrient, the maternal tissue loss of the 0.01% dietary sodium group is an example of fetal growth in the presence of maternal loss. A less severe demonstration of the same mechanism is provided by the reduced maternal growth of the rats fed 0.03% dietary sodium.

The total food intake during pregnancy (table 5) was low in the rats fed 0.01% sodium, but there were no differences among the groups fed the other levels of sodium. The smaller gains of the rats fed 0.03% sodium which were not accompanied by commensurate reductions of food intake is a result similar to that observed by Pike et al. (17) in pregnant rats fed a low sodium diet. The smaller body size, at the time of conception, of the rats fed 0.01% sodium probably contributed to their reduced food intake and may also have been a contributing factor to some of the other results with this group.

The mean food intakes and weight gains measured at 3-day intervals during pregnancy are presented graphically for the treatment groups in figure 1. A marked increase in weight gain during days 15 to 18 of pregnancy occurred in all groups, but gains were then reduced in all groups during days 18 to 21 of pregnancy. Organogenesis occurs during week 2 of gestation in the rat whereas week 3 is the period of maximum fetal growth. The increased gain during this period of maximum fetal growth was not accompanied by increased food intake in the two lower sodium groups as it was in the three higher sodium groups. The food intake during the period of days 18 to 21 was reduced from that in the preceding 3-day period in all treatment groups. Slonaker (18) noted a slight increase in the food intake of pregnant rats during the first week of pregnancy and a decrease during the final four days, whereas Murray (14) found some increase at the start of the final week of pregnancy, as was found here. Slonaker (18) studied voluntary activity of pregnant rats and found it sharply reduced throughout pregnancy.

TABLE 5

Body weights, weight change and feed intake during gestation

Dietary Na	Body weight			Change in maternal body weight [1,2]	Total food intake [1,2]
	At conception	Day 21 of pregnancy	After parturition		
%	g	g	g	g	g
0.01	181.4	211.5	175.9	−5.5 ± 2.5 [a]	268.3 ± 11.0 [a]
0.03	245.0	313.7	252.4	6.5 ± 2.8 [b]	346.2 ± 8.1 [b]
0.05	247.2	335.5	277.5	30.0 ± 3.5 [c]	370.1 ± 7.8 [b]
0.07	246.8	335.8	276.2	29.4 ± 2.8 [c]	356.8 ± 7.5 [b]
0.09	246.7	343.8	278.8	32.1 ± 5.1 [c]	365.7 ± 5.9 [b]

[1] Mean ± SEM; 9 animals in 0.01% dietary sodium group; 16 in other groups.
[2] Values with unlike superscripts are significantly different, $P < 0.01$.

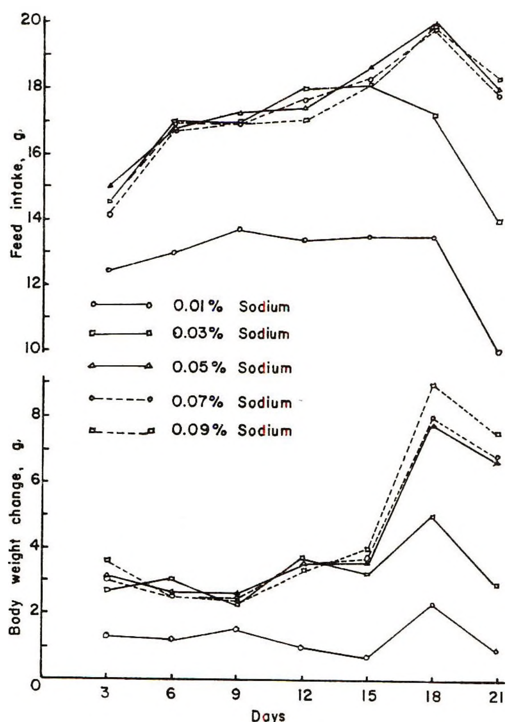

Fig. 1. Feed intake and body weight change during gestation.

Fewer pups were found in the litters from rats fed only 0.01% sodium (table 6), but no differences were detected among the other groups. Birth weights of the pups as shown in table 6 were reduced in both the 0.01% and 0.03% sodium groups and no increase occurred when the rats were fed more than 0.05% sodium. The rats fed 0.01% sodium not only had small litters of small pups, but the pups were either born dead or died very shortly after birth. Dystocia was observed in some of the rats of this group and parturition times up to 24 hours were noted. There were no maternal deaths, however. In a few instances these females were observed eating their dead pups; therefore, laparotomy was performed on some of them after parturition. Some dead fetuses were found and in each rat eight or nine fetal attachment sites were noted. It is possible that the reduced litter size found in this group (0.01% dietary sodium) was due to fetal deaths in the latter stages of pregnancy and to some of the pups being eaten at birth by the dam. Thus

TABLE 6

Litter sizes and birth weights of pups

Dietary Na	Litter size [1,2]	Birth weight [1,2]
%	*No.*	*g*
0.01	4.5 ± 0.96 [a]	4.2 ± 0.57 $_{e}^{a}$
0.03	9.8 ± 0.66 [b]	5.3 ± 0.18 $_{f}^{b}$
0.05	9.1 ± 0.74 [b]	5.8 ± 0.15 $_{g}^{b,c}$
0.07	9.3 ± 0.64 [b]	5.8 ± 0.14 $_{g}^{b,c}$
0.09	9.9 ± 0.75 [b]	6.0 ± 0.17 $_{g}^{c}$

[1] Mean ± SEM for 16 rats per group except 9 rats in the 0.01% dietary sodium group.

[2] Values with unlike subscripts are significantly different, $P < 0.05$; with unlike superscripts, $P < 0.01$.

the results suggest that this lowest dietary sodium treatment interfered with conception in an all-or-none fashion and was inadequate to maintain a full litter of viable fetuses through the final stages of gestation. The next higher dietary sodium, 0.03%, caused only a slight reduction in pup birth weights. Kirksey and Pike (5) found no reduction of litter size or fetal weight from rats fed a 0.025% sodium diet. They fed the diet only during pregnancy, however, and the fetuses were obtained by surgery.

No differences were demonstrated in the proportions of pup moisture, fat or sodium among the different dietary treatments. The mean values obtained are given in table 7. The pregnant rats fed the lowest sodium level appear to have conserved sodium by a reduction of fetal growth rather than by reducing the proportion of either sodium or water in the fetuses. An apparent bias toward selection of small pups from the 0.01% sodium group for composition analysis can be noted in the data, but this should not alter the conclusion reached.

Mean hematocrit and plasma sodium concentrations for the different treatment groups at the start and end of gestation are given in table 8. At the initiation of gestation the rats fed the 0.01% sodium diet had higher hematocrits and lower plasma sodium concentrations than were found in the rats fed more sodium. The rats in this lowest sodium group were therefore deficient in body sodium content at mating and throughout gestation. Smith and Meyer (19) found a reduced extracellular fluid volume associated with a high hematocrit in rats fed a low sodium diet even though

TABLE 7

Water, fat and sodium content of the newly born pups

Dietary Na	Dry matter per pup [1,2]	Water [1,2]	Fat [1,2]	Sodium [1,2,3]
%	*g*	%	%	*mEq/kg*
0.01	0.39 ± 0.02 [a]	86.7 ± 0.29	5.7 ± 0.20	756.3 ± 12.2
0.03	0.77 ± 0.04 [b]	86.5 ± 0.35	6.2 ± 0.24	766.1 ± 9.1
0.05	0.81 ± 0.06 [b]	86.3 ± 0.18	5.8 ± 0.26	742.2 ± 10.5
0.07	0.78 ± 0.04 [b]	86.7 ± 0.31	5.8 ± 0.25	772.2 ± 12.6
0.09	0.87 ± 0.04 [b]	86.1 ± 0.37	6.2 ± 0.28	759.4 ± 14.8

[1] Mean ± SEM for 12 pups in each group.
[2] Values with unlike superscripts are significantly different, $P < 0.01$; no significant differences among means in columns without superscripts.
[3] Expressed as mEq/kg of ether-extracted dry matter.

TABLE 8

Hematocrit and plasma sodium concentrations on days zero and 21 of pregnancy

Dietary Na	Hematocrit [1,2]		Plasma sodium [1,2]	
	Day 0	Day 21	Day 0	Day 21
%	%	%	*mEq/l*	*mEq/l*
0.01	56.6 ± 0.43 [a]	54.8 ± 1.23 $_e^a$	134.9 ± 0.78 [a]	115.2 ± 1.48 [a]
0.03	50.8 ± 0.64 [b]	49.7 ± 0.67 $_f^b$	143.6 ± 0.43 [b]	127.7 ± 1.43 [b]
0.05	51.2 ± 0.41 [b]	44.8 ± 1.38 $_g^{b,c}$	143.6 ± 0.54 [b]	133.5 ± 0.85 [c]
0.07	51.1 ± 0.47 [b]	42.8 ± 1.80 $_g^c$	143.5 ± 0.58 [b]	135.7 ± 1.10 [c]
0.09	50.5 ± 0.58 [b]	41.1 ± 1.56 $_g^c$	143.1 ± 0.47 [b]	136.7 ± 0.46 [c]

[1] Mean ± SEM for 16 rats per group except only 7 rats in the 0.01% dietary Na group on day 21.
[2] Values with unlike subscripts are significantly different, $P < 0.05$; with unlike superscripts, $P < 0.01$.

plasma sodium was only slightly low. The hematocrits determined on day 21 of pregnancy were highest in the group fed 0.01% sodium, next highest in the rats fed 0.03% sodium and lowest in the rats fed 0.05, 0.07 and 0.09% sodium, with no difference among the latter three groups. When the hematocrit values obtained on day 21 of pregnancy were compared with those obtained at the start of pregnancy, significant reductions were found to have occurred in the groups fed the three highest sodium diets, but no change was found for the two lowest sodium diets. Kirksey and Pike (5) and Pike (20) reported that the hematocrit remained about the same in pregnant and nonpregnant rats when they were fed a 0.025% sodium diet. The results of the present experiment show that approximately 0.05% dietary sodium is necessary to allow the hemodilution which usually occurs in pregnant rats. The data for day 21 of pregnancy show a trend toward increased hemodilution which usually occurs in pregnant rats. The data for day 21 of pregnancy show a trend toward increased hemodilution (decreased hematocrit) and increased plasma sodium as dietary sodium increased above 0.05% The data of Kirksey and Pike (5) indicate this trend would not have continued if dietary sodium were further increased. The plasma sodium concentration at day 21 of pregnancy was low in the group fed 0.03% sodium as well as in the 0.01% group. No difference occurred among the three higher dietary sodium groups, but all groups showed a reduction of plasma sodium during pregnancy. This reduction was much more severe in the two lowest sodium groups. The lowest dietary sodium level which permitted maintenance of normal plasma sodium was 0.05%.

The mean values obtained for bone moisture and sodium after parturition are given in table 9. The values determined for the rats fed 0.01% sodium are not comparable to those obtained for the other groups because of differences in the time when bones were removed. It appears that some bone sodium was lost after death as though it had equilibrated into total body water.

The low bone sodium found when bones were removed after storage of the carcasses was investigated in a small ancillary

TABLE 9

Bone moisture and sodium concentrations after parturition

Dietary Na	Moisture [1]	Sodium [1]
%	% *fresh wt*	*mEq/kg dry wt*
0.01	13.7±0.25	248.5±2.5
0.03 [2]	12.5±0.08	228.9±1.6
0.05 [2]	12.3±0.08	230.8±1.9
0.07 [2]	12.4±0.09	228.8±1.8
0.09 [2]	12.3±0.18	231.6±2.2

[1] Mean ± SEM, 5 rats per group except the 0.01% dietary Na group, which includes 9.
[2] Rat carcasses stored in freezer before bones were removed allowing "leakage" of Na.

trial. Six mature rats were sacrificed and one femur was removed immediately while the other was removed after the carcasses had been stored for a week in the refrigerator. All femurs were divided in half and one half was analyzed for sodium without further treatment while the other half was analyzed after it had been allowed to soak 10 times in 25 or more milliliters of demineralized water for 3 to 6 days. Small amounts of sodium were removed in the final soaking. The mean sodium content in mEq/kg dry bone for the four bone treatments were: a) removed immediately, not soaked, 278.8; b) removed immediately, soaked, 153.7; c) removed after storage, not soaked, 220.6; d) removed after storage, soaked, 142.1. These results indicate that a loss of bone sodium occurs when bones are not removed immediately after death; that this sodium is freely diffusible; and that the freely diffusible bone sodium approximates one half of the total bone sodium.

Kirksey et al. (21) found that bone sodium was decreased approximately 10% in pregnant rats fed a 0.025% sodium diet compared with those fed higher sodium levels. They also found about an 11% decrease in plasma sodium in this group during pregnancy. According to Bergstrom (22) a dynamic equilibrium exists between bone sodium and the surrounding extracellular fluid. Forbes (23) also found that hyponatremia in young rats fed a low sodium diet was reflected in a lower bone sodium content. No difference in bone sodium was demonstrated in the present data. The proportionality between bone and water found in the animal body leads to the conclusion that any large difference in bone sodium among the four higher sodium groups would have been detected despite the bone sodium "leakage."

Histological data pertaining to the kidneys and adrenals of the rats killed after parturition are presented in table 10. Data on the adrenals from the rats fed 0.01% sodium are not included because it was not possible to obtain good adrenal sections, even of 8 μ thickness, from these rats. The zona glomerulosa, however, was greatly enlarged and very diffuse, the cells showed excessive vacuolation and they appeared to have a greater number of lipid droplets. The area of the zona glomerulosa was distinctly larger in the rats fed the 0.03% sodium diet, but no differences were found among the other dietary groups.

The number of cells in the cross section of the zona glomerulosa showed a generally decreasing trend with increasing dietary sodium but a definite difference

TABLE 10

Postpartum adrenal zona glomerulosa areas, cell counts for the zona glomerulosa areas, average areas per zona glomerulosa cell and kidney juxtaglomerular indices

Dietary Na	Adrenal zona glomerulosa			Kidney juxta-glomerular index [1,2]
	Cross section area [1,2]	No. of cells per area [1,2]	Area per cell [1,2]	
%	mm^2		μ^2	
0.01	—	—	—	$29.1 \pm 2.9_e^a$
0.03	1.480 ± 0.10^a	$9898 \pm 726_e$	150.2 ± 7.5^a	$28.0 \pm 2.2_e^a$
0.05	1.000 ± 0.09^b	$7823 \pm 832_{e,f}$	128.5 ± 4.4^b	$15.3 \pm 1.2_f^b$
0.07	0.937 ± 0.10^b	$7938 \pm 654_{e,f}$	117.4 ± 4.2^b	$24.7 \pm 1.0_e^a$
0.09	0.867 ± 0.05^b	$7557 \pm 359_f$	122.6 ± 5.8^b	$21.3 \pm 1.29_g^a$

[1] Mean ± SEM, 5 rats per group.
[2] Values with unlike subscripts are significantly different, $P < 0.05$; with unlike superscripts, $P < 0.01$.

was shown only between rats fed 0.03 and 0.09% sodium. Distinctly larger cells were noted only in the 0.03% sodium group although cell areas also tended to increase as sodium decreased in the diet. Pike et al. (17) did not observe any difference in the zona glomerulosa width or number of cells from pregnant rats fed diets containing 0.025% or 0.062% sodium. An increased width of the zona glomerulosa, however, occurred in both these groups when compared with pregnant rats fed 0.14% or more sodium. The increased width of the zona glomerulosa produced by restricted sodium intake (24–26) is associated with an increased aldosterone secretion (27). Wardlaw and Pike (28) also noted a greater width of the zona glomerulosa in pregnant than in nonpregnant rats and found the width was highly correlated ($r = 0.95$) with the number of cells which comprised the width. The correlation between zona glomerulosa area and number of cells in the present study was slightly less ($r = 0.87$).

The juxtaglomerular indices tended to increase as dietary sodium decreased with the exception that the index for the rats fed the 0.05% sodium diet was distinctly smaller than for any other group. A low juxtaglomerular index was noted by Wardlaw and Pike (28) in pregnant rats fed a low sodium diet. They postulated degranulation had occurred due to the failure of the renin synthesizing ability to keep pace with secretory activity. Further support for this hypothesis was obtained by Pike et al. (17). The juxtaglomerular index data of the present study and those of Pike et al. (17) appear to be contradictory. This conflict may have been caused by the difference in the length of time the experimental diets were fed (during pregnancy only by Pike et al. (17)). Perhaps the renin synthesizing ability increases as the period of low sodium feeding lengthens. The low index found for the rats fed the 0.05% sodium diet appears real. Possibly this is an effect similar to that noted by Wardlaw and Pike (28), caused by a relatively sudden change in the sodium supply-demand relationship for this group during the final stages of pregnancy.

An inverse relationship between sodium intake and granulation of the juxtaglomerular cells was found by Hartroft and Hartroft (25) and also by Tobian et al. (29) after severe and lengthy sodium deprivation of nonpregnant rats. Hartroft and Hartroft (25) observed a direct linear correlation between zona glomerulosa width and the juxtaglomerular index. Wardlaw and Pike (28) found that this relationship was significant only when their low sodium group was omitted. The zona glomerulosa area and juxtaglomerular index were significantly correlated ($r = 0.59$) in the present study.

The data obtained here show that reproductive performance in rats is adversely affected by a diet containing 0.03% or less sodium and satisfactory when the diet contains 0.05 to 0.09% sodium.

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Proceedings of the Thirty-third Annual Meeting of the American Institute of Nutrition

SHELBURNE HOTEL, ATLANTIC CITY, NEW JERSEY
APRIL 13–18, 1969

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Saturday afternoon and evening, April 12, Sunday morning (April 13), Monday evening (April 14), and Tuesday evening (April 15). The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings, published below.

SCIENTIFIC SESSIONS

A total of 374 abstracts of papers were submitted to AIN for programing. Of these, 58 were transferred to the scientific programs of the other constituent societies; 48 were accepted from other societies, making a net total of 364 short papers for which AIN was responsible. These papers were arranged in 32 sessions (29 regular and 3 intersociety, Atherosclerosis). In addition, two conferences were held, the 34th Annual Poultry Nutrition Conference, and the 10th Annual Ruminant Nutrition Conference. Five symposia were programmed this year, one of which was televised on the closed-circuit TV channel operated during the meetings.

1. Nutrition Problems, USA, A. E. Schaefer, *Chairman.*
2. Nutrition and Cell Development. M. S. Read, *Chairman.* (Televised)
3. Nutritional Factors in the Regulation of Lipid Metabolism. G. A. Leveille, *Chairman;* A. E. Harper, *Co-chairman.*
4. Recent Developments in the Fat-Soluble Vitamins. H. F. DeLuca, *Chairman.*
5. Transition Elements in Biology. Gennard Matrone, *Chairman.*

BUSINESS MEETINGS

Business meetings were held on Tuesday, April 15 and Thursday, April 17. President R. H. Barnes presided at both meetings.

I. *Proceedings of 1968 Meetings*

The Proceedings as published in The Journal of Nutrition, 96: 163–177, 1968 were approved.

II. *Elections*

A total of 825 ballots was received. The tellers, Drs. D. K. Bosshardt and D. L. Schneider, reported the following results:

President-elect:
Alfred E. Harper

Secretary:
Doris Howes Calloway

Councilor:
John G. Bieri

Nominating Committee:
George V. Mann
Gilbert A. Leveille
Charles H. Hill
Clara A. Storvick
David Baird Coursin

III. *Membership*

As of April 15, 1969, there were 1204 members of the Institute: 1051 active, 127 retired, and 26 honorary members, this being a net increase of 62 members since last year. Fourteen members retired during the year. The following members resigned from the Institute: Drs. W. H. Daughaday, Vincent P. Dole, Karl R. Johannson, Joseph Meites, Lacy R. Overby, Robert F. Schilling, Joseph Seitchik, Leslie T. Webster, Jr., and William L. Williams. Two members were dropped from membership due to nonpayment of dues. The Clinical Division reports a total membership of 217.

Notice of the deaths of the following members was received since our last annual meeting:

Anne B. Caldwell, December 3, 1968
Cornelia Kennedy, January 13, 1969
Laurance W. Kinsell, July 10, 1968
Robert C. Lewis (Charter Member), February 23, 1969
Agnes F. Morgan (Charter Member), July 19, 1968
William N. Pearson, November 28, 1968
Elaine P. Ralli, October 6, 1968
Mary Elizabeth Reid, October 7, 1968
Adelaide Spohn (Charter Member), July 17, 1968
Jakob A. Stekol, January 17, 1969
William McLean Wallace, November 9, 1968

The Council of the American Institute of Nutrition adopted the following resolution.

RESOLVED: That the American Institute of Nutrition, assembled at its annual meeting in Atlantic City, N. J., April 15, 1969, wishing to recognize the loss by death during the past year of three of its distinguished Charter Members, ordered that the following statements be placed in the Proceedings as a permanent record.

Robert Curtis Lewis was trained at Yale University, receiving the Ph.B. degree in 1909 and the Ph.D. degree in 1912 under Professors Lafayette B. Mendel and F. W. Underhill. Dr. Lewis' professional career included teaching, research and administration and began at Yale University as a student assistant in 1909. From 1912 to 1914 he was Research Physiologist at General Memorial Hospital in New York City. From 1914 to 1916 he was Assistant Biochemist with the United States Public Health Service. Then for a 40-year period he was a member of the faculty of the University of Colorado School of Medicine as Chairman of the Department of Biochemistry (1916–1948) and as Dean of the School of Medicine and Associate Dean of the Graduate School (1948–1956). Following his retirement from the University in 1956 as Dean Emeritus and Emeritus Professor of Biochemistry, he served as a Consultant in Biochemistry to the Department of Pathology at the Colorado State Hospital in Pueblo until 1964, when health difficulties limited his activities.

Dr. Lewis' primary research interests were clinical blood chemistry, carbohydrate and mineral metabolism, and the vitamin B-complex. One of his early papers was with Stanley R. Benedict in 1915 describing a quantitative method for the determination of blood sugar by a color reaction with picric acid. With J. M. Orten and Aline Underhill (Orten) in 1932, he was the first to confirm the work of the Wisconsin group on the essentiality of copper for hemoglobin formation in the rat. In the mid-30's he also published a number of papers on members of the vitamin B-complex.

Dr. Lewis was a member of a number of scientific societies and served as an officer in several. He enjoyed particularly informal talks with his colleagues and friends. Dr. Lewis was indeed one of the great teachers of nutrition and biochemistry of our time as well as a warm friend and an inspiring mentor.

Agnes Fay Morgan was a charter member of the American Institute of Nutrition. She was active in its affairs from the time of its founding until her death in August 1968, at which time she was serving as Chairman of the Fellows Committee of the Institute. She received the Borden Award of the Institute in 1954, and was elected a Fellow in 1959.

Dr. Morgan was born in Peoria, Illinois in 1884. She went first to Vassar and then to the University of Chicago, where she graduated as B.S. in chemistry in 1903. Her graduate education was also at the University of Chicago, where she received the Ph.D. degree in 1914, with a chemistry major, after an interlude of teaching chemistry at Hardin College, the University of Washington, and the University of Montana. She came to Berkeley as assistant professor of nutrition in 1915, and set to work to build a department of Home Economics, of which she was chairman from 1915 until her retirement in 1954. Following this, she continued her research in the department, which now bears the name of the Department of Nutritional Sciences. In 1958, she moved with the department into the large building of the Berkeley campus which was named Agnes Fay Morgan Hall in 1961 by the University of California. Here she worked daily in her office, with a fine view of her beloved San Francisco Bay, until the summer of 1968.

Dr. Morgan's research interests were wide-ranging. She is well known for her pioneer studies on the effects of heat on the nutritional value of proteins, and on the effects of vitamin-B-complex deficiency on experimental animals. She also made important findings in the fat-soluble vitamin field, in nutritional physiology, and in the vitamin content of prepared foods. Her most recent work was a history of the American Institute of Nutrition, which she presented at the 1968 Federation Meeting. Among her numerous honors, in addition to those mentioned above, were the Garvan Medal of the American Chemical Society (1949), the Faculty Research Lectureship of the University of California (1951), LL.D. California (1959), Fellow of the University of California (1968), and Professional Achievement Award, University of Chicago (1968). In 1965, a symposium was held at the University of California in celebration of her first 50 years of research.

Perhaps her greatest contribution to science was the influence of her indomitable and sunny personality on a host of students and fellow scientists, on nutrition education, and on the progress of the science of nutrition.

Born in Chicago, May 25, 1886, Adelaide Spohn received the degrees of B.S. and M.S. from the University of Chicago and, from Columbia in 1922, the degree of Ph.D. She then was named assistant professor, later

professor, of home economics at Cornell University until 1931. The year 1930–1931, however, and the following year, were spent as visiting fellow at Yale. Dr. Spohn then returned to Chicago, and was employed as a nutritionist by the Elizabeth McCormick Fund until her retirement in 1952. For the next five years she was on the full-time staff of Mercy Hospital in Chicago, where she established nutrition services in some of the out-patient clinics, a type of activity which long had claimed her attention. In 1957 she removed to Summit, New Jersey, to be near a sister who survives her.

Miss Spohn was a soft-spoken woman, reticent almost to a fault. Few of her friends realized that she had begun her career as a teacher of chemistry, and that she had worked for four years with Dr. Oscar Riddle, both in Chicago and at Cold Spring Harbor, on the chemical aspects of reproduction in birds. At Columbia University, under Professor H. C. Sherman, she had turned her attention to food chemistry, particularly to the study of quantitative methods of assay for different vitamins, and the effects of processing on the nutritive value of foods. But it was as a nutritionist that Miss Spohn went from Yale to the McCormick Fund, and it is as an organizer of continuing programs of nutrition education that she will be best remembered.

Her reports to and from the McCormick Fund were submitted in the name of the organization with no individuals mentioned. The Fund, which continues to operate as a separate organization now under the direction of the Chicago Community Trust, would "loan" Miss Spohn to selected organizations and agencies for a specific purpose, and pay her salary and the expenses of her activities. For several years during the depression, Dr. Spohn directed the nutrition advisory services of the Illinois Relief Commission, for whom she developed a budgetary plan for families on relief which, in its essential features, is still followed. Hardly had this activity been completed to a point where the State could take over, when World War II erupted and made new demands on education in nutrition. Miss Spohn was loaned to the Chicago Board of Health, where again she pioneered in establishing, with the help of assistants whom she had trained, a continuing educational program in nutrition. Dr. Spohn's accomplishments as a team-oriented leader are difficult to separate from the total accomplishments of the organizations she served. The record of her career is best read, not in books or journal articles, but in the lives of the people of her City and her State.

The members of the American Institute of Nutrition present at the annual meeting in Atlantic City, N. J., on April 15, 1969, unanimously passed a motion directing that the following obituary notice concerning Dr. W. N. Pearson be prepared and placed in its Proceedings as a permanent record expressing regret and deep sorrow at the loss of this talented member at the height of his career by his tragic death on Thanksgiving Day, November 28, 1968.

WILLIAM NORMAN PEARSON

On Thanksgiving Day, November 28, 1968, Dr. William N. Pearson and his 12-year old son, Christopher, were killed in an automobile accident while en route to the annual Thanksgiving Day football game. His death was a loss to the science of nutrition, but an especially great one to the American Institute of Nutrition. At the time of his death he was completing his third year as Secretary of the AIN. Since 1966 he had served on the editorial board of The Journal of Nutrition, and was to have assumed the editorship of the Journal on January 1, 1969.

Prior to becoming Secretary of the AIN, Dr. Pearson had served (1963–1966) as chairman of its international nutrition committee. He was general chairman of the highly successful Western Hemisphere Nutrition Congress II, held in San Juan, Puerto Rico, August 25–29, 1968, which was co-sponsored by the Council on Foods and Nutrition of the American Medical Association, the American Institute of Nutrition, the Canadian Nutrition Society and La Sociedad Latinoamericana de Nutricion.

These contributions to the AIN were but one facet of this remarkably versatile and talented man. At the time of his death he was serving as a member of the NIH study section on nutrition, of the Committee on Recommended Dietary Allowances of the Food and Nutrition Board, of a committee of the Council on Foods and Nutrition of the AMA, and as special consultant to the nutrition program, National Center for Chronic Disease Control, PHS (formerly the Interdepartmental Committee on Nutrition for National Development).

To the latter organization he made immeasurable contributions, serving as laboratory director of the ICNND nutrition surveys in the Philippines, Spain, Ecuador, Paraguay, Lebanon, Jordan, and Trinidad. The experience made him one of those most knowledgeable concerning the use of biochemical measurements for assessing human nutritional status.

Dr. Pearson was born on January 5, 1924, in Butler, N. J. After service in the army during World War II, he obtained his B.A. degree from Tusculum College in 1947, and his Ph.D. from Vanderbilt University in 1951. Throughout his postdoctoral career he held faculty positions in the Department of Biochemistry and Division of Nutrition at Vanderbilt, rising in rank from instructor to professor and associate director of the Division of Nutrition in 1967. He was an excellent teacher and a resourceful and productive investigator. He received the AIN Mead Johnson Award in 1967 for his research contributions, in particular for those dealing with the metabolism of thiamin. His personal qualities of keen intellect, diligence, and quick wit engendered

respect and affection in his students and associates.

[Much of the above was taken from a fuller obituary notice by W. J. Darby which appeared in AIN Nutrition Notes, Volume 5, Number 1, March 1969.]

IV. *New Members*

The Membership Committee considered the qualifications of 112 nominees. The following 92 nominees recommended by the Council were approved for membership at the business meeting:

NEW MEMBERS — 1969 *

Adibi, Siamak A.
Adkins, James S.
Ahluwalia, Balwant S.
Anthony, W. Brady
Baker, David H.
Barney, George H.
Baugh, C. M.
Bayley, H. S.
Brinkman, G. L.
Bronner, Felix (C)
Brunser, Oscar (C)
Bunnell, R. H.
Campbell, T. C.
Carter, James P. (C)
Chopra, Joginder (C)
Daghir, N. J.
Davidson, K. L.
Dawson, E. B. (C)
Drenick, E. J. (C)
Dua, P. N.
Eidelman, S. (C)
Finkelstein, J. D. (C)
Fuqua, M. E.
Gailani, S.
Giorgio, A. J. (C)
Glinsmann, W. H.
Gordon, J.
Grummer, R. H.
Guthrie, H. A.
Hankin, J. H.
Hansen, H. J. (C)
Hanson, R. W.
Hatfield, E. E.
Hill, D. C.
Hill, R. B.
Hoerman, K. C.
Huber, J. T.
Irwin, M. I.
Jacobson, D. R.
Jeejeebhoy, K. N. (C)
Jeffries, G. H. (C)
Jelliffe, D. B. (C)
Kakade, M. L.
Kelsay, June
Kenney, M. A.
Klein, Robert (C)
Law, David H. (C)
Lees, Robert S. (C)
Levy, Robert I. (C)
Little, C. O.
Lombardi, B.
Lowenstein, F. W.
Luick, J. R.
Marusich, W. L.
Matschiner, J. T.
McCoy, E. E.
McLaughlan, J. M.
Mitchell, G. E., Jr.
Noland, P. R.
Norman, A. W.
O'Brien, William (C)
Parks, P. F.
Pleasants, J. R.
Pope, A. L.
Reid, R. L.
Richardson, K. E.
Robinson, D. W.
Roe, D. A.
Rosenthal, H. L.
Rosenthal, W. S.
Sanbar, S. S. (C)
Sardesai, V. M.
Schenk, E. A. (C)
Shapiro, R.
Slinger, S. J.
Sodhi, H. S.
Stillings, B. R.
Stowe, H. D.
Streiff, R. R. (C)
Travis, H. F.
Tripathy, K. (C)
Van Soest, P. J.
Vander Noot, G. W.
Waldroup, P. W.
Weber, C. W.
Winchester, C. F.
Woodard, J. C.
Wurtman, R. J. (C)
Zakim, David (C)
Zee, Paulus (C)
Zeman, F. J.
Zilversmit, D. B.

* For institutional affiliations and addresses of new members, see the Federation Directory of Members, 1969.

(C) Also elected to membership in the Clinical Division, the American Society for Clinical Nutrition, at its Annual Meeting, May 3, 1969.

The following AIN members were elected to membership in the Clinical Division: T. E. Huber and Peter D. S. Wood.

HONORARY MEMBERS

The following distinguished scientists were elected to Honorary Membership in the AIN:

Dr. Egon H. Kodicek, *Director of Dunn Nutrition Laboratory, University of Cambridge, England.*

For his many contributions to the advancement of the knowledge of nutrition, and as a stimulating teacher of young scientists from many countries. Through his leadership in clinical nutrition at Charles University, Prague, then for more than two decades at Cambridge University, and as a member of international committees and commissions he has provided leadership and guidance leading to better health of people in many countries.

Dr. Thomas Moore, *Strangeways Research Laboratory, Cambridge, England.*

For his many contributions to our knowledge of the metabolism of vitamins A and E over the past 40 years. He showed conclusively that β-carotene was transformed to vitamin A in the body and demonstrated the sparing effect of vitamin E on vitamin A. During his long association with the Dunn Nutrition Laboratory he developed many aspects of vitamin A and E in the nutrition and physiology of animals and man. His book "Vitamin A" published in 1957 remains a classic reference even today.

Dr. M. Swaminathan, *Chairman, Applied Nutrition and Dietetics Discipline, Central Food Technological Research Institute, Mysore, India.*

For his many contributions to nutritional research and to improved public health through nutritional innovations in his native India. He has developed assays of important vitamins, determined the chemical composition of Indian foodstuffs and developed low-cost protein foods for treatment and prevention of protein malnutrition in children. In addition to reporting the results of his numerous original researches, he has written valuable books on nutrition, was founding editor of the Journal of Nutrition and Dietetics and has served on important international committees.

Dr. Hiroshi Morimoto, *Head, Department of Nutrition, National Institute of Animal Industry, Chiba-Shi, Japan.*

For his many contributions in the field of animal nutrition in Japan for the past 30 years for which he received the commendation of the Japanese Ministry of Agriculture and Forestry in 1967, for his leadership as head of the nutrition research division (now designated the Department of Animal Nutrition) of the National Institute of Animal Industry of Japan, for his numerous public services, particularly as an official government representative on international matters in animal production, and as the author of several books on animal nutrition which have helped greatly in the development of the Japanese livestock industry.

Professor Richard A. Morton, *Johnston Professor of Biochemistry, University of Liverpool, England.*

For his signal contributions to the biochemistry of lipids and his long service in the interests of nutrition research, teaching and public

education. Through his membership on committees of The Royal Society, Biochemical Society, Nutrition Society, Royal Institute of Chemistry and many other bodies, he has provided distinguished leadership in the development of new knowledge in nutrition, the training of young scientists and the implementation of public health nutrition programs.

V. *President's Report*

AIN Forum. Plans were confirmed for the open forum scheduled to be held later during this meeting to encourage members of both AIN and ASCN to present their views concerning the objectives that our professional society should have. Major attention will be given to the important questions concerning the appropriateness of membership eligibility rules and practices, and the role of AIN in the establishment and execution of national and international nutrition policies and programs. The forum will provide an opportunity for any member to introduce subjects dealing with the operation of the AIN.

A committee representing the different professional interests of the membership has been established to provide AIN Council with recommendations concerning the scope and function of AIN. These recommendations will be drawn from the deliberations of the forum and from other sources of information and suggestions available to the committee. The members of the committee are Drs. R. H. Barnes, J. F. Mueller, D. C. Cederquist, D. E. Becker and Lavell Henderson.

Council Actions. Council approved AIN's participation in interdisciplinary symposia for the first annual National Biology Congress scheduled to be held in 1970 in Detroit, Michigan. This Congress is sponsored by the American Institute of Biological Sciences (AIBS) for the purpose of encouraging the involvement of young scientists, including local high school and college students, and to stimulate communication between scientists and the lay public.

Council approved AIN co-sponsorship of the Third Western Hemisphere Nutrition Congress to be held in 1971 in Florida.

AIN and ASCN have agreed to form a joint committee consisting of members appointed from each of the two societies to consider and, if feasible, to plan a joint meeting of the AIN and ASCN. This meeting would be separate and in addition to the regular Annual Meeting held in conjunction with the other Federated societies.

Council accepted an offer made by Mrs. Wendell H. Griffith for the establishment of a Wendell Griffith Memorial to be used to support, in part, selected AIN symposia.

VI. *Federation Affairs:* B. L. O'Dell

New FASEB Committee, Long Range Planning Committee. As the result of recent deliberations by both the FASEB Executive Committee and the Board, it was decided that an ad hoc Long Range Planning Committee be established with Dr. L. D. Carlson as chairman. The committee is composed of one representative and one alternate from each of the six societies. The AIN Council has appointed Dr. W. J. Darby as representative and Dr. Hartley W. Howard as alternate. The first meeting was May 15 and 16. Agenda items submitted by the AIN Council included the questions of the Federation and its relation to the constituent societies; justification of certain staff functions within the Federation (Office of Public Affairs, Office of Biomedical Studies), and in contract activities with Federal agencies; and procedures for more equitable disbursement of Federation income among the societies from such sources as annual meeting registration fees and exhibit space rental fees.

Federation Proceedings (FP). During the February meeting of the FASEB Executive Committee there was considerable discussion on possible changes in FP, but motions to remove the abstracts from the publication, to change its name or the number of issues published each year were tabled. Prior action by an ad hoc committee increased the domestic nonmember subscription rate from $12 to $20 (with proportionate increase for foreign subscriptions) with no increase in the special $6.00 member rate.

Approval for publication of letters to the editor and papers on public policy related to science was granted. It was confirmed that all articles offered for publication in FP, including those arising from symposia or general sessions of the annual meeting, are subject to review and approval

by the Editorial Committee which is responsible for all the text material in FP.

FASEB/AIBS. Last year at the April meeting, the FASEB Board approved the establishment of an ad hoc coordinating group composed of the Past Presidents, the Presidents, and the Presidents-elect of both FASEB and AIBS to promote collaboration on mutual problems in public policy. This committee met in November and chose the name, *American Biology Council.* They recommend that two members-at-large be added and that a joint newsletter be instituted. Mr. Robert Grant, Director of the FASEB Public Affairs Office, plans to work closely with the editor of the AIBS Newsletter.

VII. *Executive Secretary's Report*

VIIIth International Congress of Nutrition, Prague, Czechoslovakia, August 28–September 5, 1969. The AIN Travel Grant Committee, a committee of the U.S. National Committee, IUNS, expects to disburse about 100 grants from the total of $35,000 available ($15,000 from AIN, $15,000 from the National Institute of Arthritis and Metabolic Diseases, and $5,000 from the National Institute of Child Health and Human Development). The National Science Foundation did not approve the AIN application for additional funds for travel to Prague.

The amount of each grant is based on the location of the awardee and the cost of travel from point of departure to Prague and return. Grants this year range from $325 to $450 and can be used to support travel only.

The Committee, consisting of Drs. James Waddell, O. L. Kline, G. F. Combs, J. G. Bieri, and R. Van Reen, has identified recipients from the large number of applications on the basis of choosing those U.S. scientists most importantly involved in the Prague program. The Committee will support also two of the three official delegates representing the U.S. at the Congress (the National Academy of Sciences will provide funds for the third delegate).

Two charter flights are being planned by the Chevy Chase Travel, Inc., and all transportation information is being sent to travel grant recipients, AIN members and others who indicate any interest in attending the Congress. In the event that the charter flights are cancelled due to insufficient number of reservations, group flights will be offered. [Note: Both charter flights had to be cancelled in May, 1969.]

VIII. *Treasurer's Report*

The 1968 Financial Statements, reproduced here, were presented by Mr. John R. Rice, AIN Business Manager, and accepted and approved. The AIN Auditing Committee, Drs. A. E. Light and D. A. Benton, reported that they had examined the financial report and the records in the AIN business office and found them to be in order.

The recommendation of the Council that the AIN dues remain the same for 1969–1970 ($7.00) was approved by the membership.

IX. *Report from the Clinical Division*

All AIN and ASCN members have received the outline of the program and an invitation to attend the ASCN Annual Meeting to be held in Atlantic City, New Jersey on Saturday, May 3, 1969. Formal presentation of the McCollum Award will be made during a luncheon this year to Dr. Edward H. Ahrens, Jr., of Rockefeller University.

The ASCN has been able to locate the 1969 meeting in the Haddon Hall, the hub and focus of all of the clinical meetings held in early May. The program of the ASCN meeting appeared in the program of the American Federation for Clinical Research which made this program available to about 5,000 clinicians.

The scientific program consists of the symposium, Nutritional and Hormonal Control of Gluconeogenesis, chaired by Dr. A. B. Eisenstein, and an afternoon session of short papers.

The ASCN Council, at its November 1968 meeting, approved the increase in the member subscription rate for The American Journal of Clinical Nutrition from $11.50 to $15.00, an increase in the nonmember subscription rate to $20 domestic (postage additional for foreign subscriptions) and approved the institution of a page charge to become effective in July

Financial Statement — December 31, 1968

ASSETS		EXHIBIT A
Cash		$ 35,894
Accounts Receivable		11,384
Investments		111,037
Furniture and Equipment	$ 4,275	
Less Accumulated Depreciation	(1,050)	3,225
Journal of Nutrition (Cost)	$62,500	
Less Accumulated Amortization	(3,125)	59,375
Prepaid Expenses		5,755
Total Assets		$226,670
LIABILITIES AND FUND CAPITAL:		
Accounts Payable		$ 44,050
Long Term Debt (Wistar Institute)		50,000
Fund Capital		132,620
Total Liabilities and Fund Capital		$226,670

Statement of Revenue and Expense and Fund Capital
For the Year Ended December 31, 1968

EXHIBIT B

Revenue:	TOTAL	GENERAL FUNDS	SPECIAL FUNDS
Membership Dues and Contributions	$ 7,487	$ 7,487	$ —
Sustaining Associate Memberships	7,400	7,400	—
Annual Meeting Registration	5,987	5,987	—
Journal of Nutrition:			
Subscriptions and Single Issue Sales	92,634	92,634	—
Reprints	15,621	15,621	—
Advertising	2,124	2,124	—
Excess Illustrative and Tabular Charges	10,427	10,427	—
Page Charges	22,205	22,205	—
Interest	8,495	5,456	3,039
Grants	30,900	—	30,900
Overhead	7,522	7,522	—
Miscellaneous	459	276	183
Total	$211,261	$177,139	$ 34,122
Expenses:			
Salaries, Payroll Taxes, and Fringe Benefits	$ 24,118	$ 23,550	$ 568
Communications, Postage and Travel	12,447	10,733	1,714
Supplies and Duplicating	4,420	4,239	181
Travel Awards	24,105	579	23,526
Journal of Nutrition Editor's Office	13,096	13,096	—
Rent Expense	3,169	3,169	—
Depreciation and Amortization	3,548	3,548	—
Printing and Engraving	122,141	122,141	—
Miscellaneous Expenses	4,787	950	3,837
Total Direct Expenses	211,831	$182,005	$ 29,826
FASEB Business Service Charge	1,944	1,350	594
Total	$213,775	$183,355	$ 30,420
Revenue Over Expenses	($ 2,514)	($ 6,216)	$ 3,702
Fund Capital:			
Balance, 12/31/67	135,134	109,624	25,510
Balance, 12/31/68 — To EXHIBIT A	$132,620	$103,408	$ 29,212

1969. These steps were judged necessary by the Publications Management Committee and the Council to defray an anticipated deficit of the operation of the journal.

X. *Editor's Report — Journal of Nutrition*

It was announced that, effective July 1, 1969, Dr. Fredric W. Hill will succeed Dr. Richard H. Barnes as Editor of The Journal of Nutrition. The proposed, initial budget submitted by Dr. Hill covering the period July–December 1969 was approved and is included below.

BUDGET

Journal of Nutrition Editorial Office
July–December 1969

Salaries:	
Editorial Assistant (incl. 10% fringe)	$4,730
Typist-Clerk (half time — 10% fringe)	—
Communications (telephone, postage)	900
Office Supplies	400
Travel Expense	400
Office Equipment	1,500
(To equip office for editorial assistant with desk, table, files, chair, typewriter, bookcase, supply cabinet. Also dictation transcribing unit for typist)	
Renovations	1,000
(Share of cost of renovating office space, required to accommodate Journal staff)	
Other Expense	100
Overhead to University of California	430
Total	$9,460

Dr. Barnes gave recognition and thanks to the outgoing members of the Editorial Board and special thanks to Mrs. Kathleen Berresford, who assisted him so competently during the past ten years.

Dr. Barnes' report on manuscripts processed during the past year appears below:

A motion from the floor was unanimously passed directing that the following resolution expressing appreciation of Dr. Barnes' services as Editor of The Journal of Nutrition, be placed in the Proceeding for permanent record.

RESOLVED: That the American Institute of Nutrition express its gratitude and recognize the debt of the members to Dr. Richard H. Barnes for his service as Editor of The Journal of Nutrition from September 1959 to July 1969. During these ten years the Journal format was changed to permit publication yearly of more papers but with fewer total pages. The editorial board was expanded from 12 to 20 members and a Biographical Editor and an Associate Editor were added. As a consequence of the dedicated Editorship of Dr. Barnes, the Institute now owns a Journal which has retained and enhanced its scientific stature while becoming a more efficient repository of the expanding experimental literature of nutrition. Thus, as professionals we can appreciate the personal sacrifice required and the responsibility imposed for an Editor to provide the leadership needed to maintain the scientific standards and reputation of the Official Organ of the Institute. We are deeply grateful to Dr. Barnes for leaving a journalistic legacy in which all can take pride.

XI. *Reports of Committees and Representatives*

A. *Publications Management Committee*: W. J. Darby, Chairman.

During the past year we have accumulated initial experience in managing our newly acquired Journal of Nutrition. Publishing functions have been moved as of January 1969 to AIN headquarters, with business and redactory services being performed by contract with the FASEB Office of Business Services and the Office of Editorial and Information Services, respectively. The Wistar Press now serves as the printer of the Journal.

Editing and Publication Operations (Calendar Year)

	1966	1967	1968
Volumes published	88,89,90	91,92,93	94,95,96
Pages published — total exclusive of supplements	(not recorded)	1762	1819
(Scientific papers)	(1350)	(1606)	(1697)
(Biog., AIN business and Letters)	(97)	(72)	(122)
(Index, contents, covers, etc.)	(not recorded)	(84)	
Papers published (including 3 biographies)	194	225	233
Papers submitted	316	326	344
Papers rejected during year regardless of when submitted	89	95	111
Supplements published	0	2	0
Letters to the Editor	0	1	0

Your Committee, the AIN Council and staff and John Rice, our good business manager, have been constantly alert to the matters of cost incurred in this new arrangement. We have searchingly reviewed these costs for last year, preliminary estimates of costs for the initial numbers of 1969 (January and February issues), and the projected budget. These initial costs are, in our judgment, subject to downward adjustment during the present year. Furthermore, we are encouraged that there is a prospect of greater income to the Journal from page charges and reprints under our new rate schedule and management policy.

We are, under instructions from your Council, proceeding with further explorations of means to increase the income and decrease the cost of operation of The Journal of Nutrition. We shall report to your Council on some further aspects of the financial outlook by June 1, 1969. During the year we assure you that we shall repeatedly reassess the financial position of the Journal in order to assure the best financial position possible during this year — and hopefully we may he able to report to you next year tbat the Journal has operated in the black.

As Chairman of the Publications Management Committee I wish to convey to our effective and loyal Editor (and President) Dick Barnes and his staff our heartfelt thanks for these many years of editorial leadership and for the most pleasant working relationship which has existed between the Editorial Office and the Publications Management Committee. During this period we have acquired the ownership of the Journal and realigned our overall management. We welcome Fred Hill as the new Editor and assure him our fullest support. During his Editorship I am confident that we can look forward to establishing a firm financial and managerial base.

B. *Committee on Experimental Animal Nutrition*: G. F. Combs, Chairman.

This Committee has continued to represent the experimental animal nutritionists in the affairs of AIN. Two conference programs were held at the annual meeting: the 34th annual poultry nutrition conference and the 10th annual ruminant nutrition conference.

The subcommittee chaired by Dr. D. C. Church has reduced its collection of slides depicting nutritional deficiencies in experimental animals to approximately 500 slides. The subcommittee is now ready to prepare a catalogue listing these after further screening, together with black and white prints of each. Approximately ten sets of slides, divided by nutrient, species and tissue, will be reproduced and made available to the AIN. Also, arrangements will be made so that these slides can be purchased by number from a commercial agency. Means of funding these activities are being explored. [Council approved the Committee's proposal and authorized the Executive Secretary to work with the Committee on Experimental Animal Nutrition to seek outside support for the publication of an illustrated catalogue offering sets, individual, or the complete collection.]

The members of the subcommittee are: Dr. D. C. Church, Chairman; Drs. R. W. Luecke, W. H. Pfander, K. Harshbarger, L. Potter, L. E. Harris, L. H. Breuer, and G. F. Combs.

New appointments to this Committee appear in the list of committees following these Proceedings.

C. *Public and Professional Information Committee*: M. S. Read, Chairman.

The Public and Professional Information Committee met on Saturday, April 12, 1969 in Atlantic City. It was noted that during the past year all Committee members have participated fully and actively in the various projects of the Committee. One project was completed, the Report on Booklists, and press releases were prepared for AIN award recipients and new Fellows, and newsworthy papers for the Annual Meeting were identified and selected for press coverage.

In reviewing the committee's responsibility for interpreting nutrition research through public and professional media, it was agreed that a new effort should be made to identify newsworthy articles appearing in the AIN journals. In view of the changing editorship for The Journal of Nutrition, it was agreed that efforts at the present time might best be focused on The American Journal of Clinical Nutrition. Dr. R. Bradfield, as a member of the editorial board for the AJCN, volunteered to assist the editor in identifying articles for special attention. Following a one-year pilot effort, the committee will review (April 1970) whether this activity can be expanded to further serve The Journal of Nutrition as well.

Science Project Subcommittee

A "mock-up" of a brochure entitled "Guide to Nutrition Science Projects" was prepared by the science project subcommittee and presented to the full committee for review and comment. The proposed brochure will be designed to fit a standard business envelope. It was reported that this brochure can be printed in three colors in a pilot run in Blacksburg, Virginia for $250 for the first 1,000 copies or $900 for 10,000 copies.

The revised text was briefly reviewed by the committee and individual comments were given to Dr. Ackerman. A revised text will be prepared by Dr. Ackerman for pilot testing before a final draft is printed for national use.

Dr. Ackerman suggested that the pilot testing program include distribution of a copy of the brochure to all AIN members for comment and preliminary use in their community. An additional 2,000 copies will be tested in the Virginia high schools during 1969–1970 school as follows:

> In September 1969, copies will be sent to high school science teachers in one-half of the schools that are members of the Virginia Junior Academy of Science Program. Additional copies will be sent to high school teachers in schools that are not members of the Junior Academy Program, but which participate in the State Science Fair Program. The

schools which did not receive a copy of the brochure will serve as controls. The number and kind of science projects that exhibit at the Junior Academy Meetings and the Science Fairs in the spring 1970 will be evaluated and compared to the number of exhibits shown in previous years, for which records are available. Comments of the science teachers concerning use and clarity of presentation will also be obtained by questionnaire.

If the results of this test indicate the brochure was effective, plans will be made to obtain support for a national program with science teachers.

[In reviewing the above proposals, Council endorsed the activities of the subcommittee. One thousand dollars have been reserved for the pilot test program. Dr. Ackerman was instructed to proceed with a revision of the text and the proposed test program.]

Career Brochure Subcommittee

Dr. Bradfield reported that the career brochure subcommittee had developed two alternate revisions of the brochure for high school students. The text of these brochures was tested in the Berkeley, California high school and revisions made in line with the student comments. It is interesting that the students preferred a socially oriented draft, whereas mature nutrition scientists preferred the alternate, more detailed version. The full committee reviewed both drafts, and, based on these comments, prepared near-to-final copy which they recommend be substituted for the present career brochure.

[In reviewing this proposal, Council endorsed the revised text and recommended that AIN move forward in designing a new brochure based on this text. Should any final revisions need to be made by subcommittee members, this should be handled through Dr. Bradfield who will be in charge of final copy. Use of the new brochure will be undertaken as soon as the supply of the present version is exhausted.]

The committee also reviewed the need for a career brochure aimed at university and graduate students. It was noted that this would cost considerably more than had been previously anticipated, even to produce the relatively small numbers needed for limited placement. Furthermore, the committee recognized the time which would be required of committee members or alternately for the hiring of a special science writer to write, design, and publish a brochure having the desired quality. After thorough discussion, the committee concurred that it doubted whether such a brochure would achieve useful goals and motivate university students to enter a nutrition research field.

As an alternative, the committee recommended that the AIN identify all university research training centers in nutrition in the United States, drawing upon the experience and knowledge of AIN members. Such a compilation would be valuable for directing graduate students to suitable locations for professional training.

[Council was in agreement as to the need and potential usefulness of such an index. The committee was encouraged to move forward aggressively on this project and to report back to Council next year.]

Survey of Membership Knowledge of Federal Nutrition Programs

Dr. Bradfield reported the results of the survey of AIN membership concerning Federal food and nutrition programs. It was noted that 97% of the respondents recommend that a special effort be made to bring information concerning present programs and changes in these programs to the membership through Nutrition Notes. The committee recommends that appropriate officials in responsible government agencies prepare these brief summaries for publication in Nutrition Notes.

[Council endorsed the recommendation of the subcommittee that Nutrition Notes should include a regular systematic review of Federal nutrition programs in order that the membership may be fully informed.]

D. *Committee on Nomenclature*: S. R. Ames, Chairman.

Organizational

The 1968–1969 Committee on Nomenclature was activated by President Barnes at the 1968 AIN meeting at Atlantic City with Drs. E. W. Crampton, P. L. Harris, Q. R. Rogers and H. E. Sauberlich as members. Drs. C. G. King and S. R. Ames continue as members of the Committee on Nomencalture of IUNS. Liaison with IUPAC-IUB was continued through Dr. Waldo Cohn, Director, NAS–NRC Office of Biochemical Nomenclature. The Committee met at the 1968 meeting at Atlantic City and is planning to meet on April 14, 1969 at Atlantic City.

Implementation of Committee Recommendations

The AIN Committee on Nomenclature recommended to the Council at the April 1968 meeting that the Committee's recommendations be accepted as the official nomenclature of AIN. The Council accepted this recommendation and further recommended to the editorial boards of the official journals that these recommendations be implemented as the nomenclature of the official journals of the AIN (J. Nutr., 96: 168 (1968)).

Vitamin Nomenclature

Tentative Rules for the "Nomenclature of Cyclitols" (including myo-inositol) have been issued by joint action of IUPAC-CON and CBN.

A "Third Draft" of "Tentative Rules for Generic Descriptors and Trivial Names for Vitamins and Related Compounds" was reviewed by this Committee at the annual meeting on April 16, 1968. Copies were widely circulated including the editorial board, Journal of Nutrition; editorial board, American Journal of Clinical Nutrition;

IUNS Committee on Nomenclature; and IUPAC-IUB Commission on Biochemical Nomenclature. The "Third Draft" was reviewed by IUPAC-IUB-CBN at a meeting held in Bellagio, Italy, June 10–12, 1968 with Drs. H. Dam and T. Moore present as IUNS observers. Drs. S. Ames and T. Moore held an informal conference at the Vitamin A Symposium held at M.I.T., November 25–27, 1968. Based in part on the comments received from representatives of the above organizations, a revised "Fourth Draft — February 28, 1969" was prepared. The terminology in this "Fourth Draft" is consistent with the nomenclature used by the Food and Nutrition Board, NAS–NRC, in their VIIth Edition, 1968 of "Recommended Dietary Allowances."

The "Fourth Draft — February 28, 1969" of the AIN Committee on Nomenclature's proposed "Tentative Rules for Generic Descriptors and Trivial Names of Vitamins and Related Compounds" was submitted to the Council, American Institute of Nutrition for approval at the 1969 Atlantic City Meeting. We hope that following Council approval, these proposed "Tentative Rules" can be immediately adopted by U.S.A. nutrition journals. Future revisions will be dependent on acceptable proposals by the IUNS Committee on Nomenclature.

Calorie versus Joule

In 1948, the 9th General Conference on Weights and Measures adopted the "joule" as a unit for electrical work, heat, mechanical work and energy, thus avoiding the calorie as far as possible. ISO (International Organization for Standardization) has recommended (R-31, Part III) the adoption of "joule" as the preferred unit and these recommendations have been adopted by National Bureau of Standards. These actions reflect the international adoption of an "International System of Units" in which there is a single unit for each physical quantity.

One I.T. calorie is equal to 4.1868 joules. The impact of the adoption of the "joule" on the status of the "calorie" and on nutritional science is currently under review by this Committee.

Chairman's Comments

Substantial progress has been made in establishing preferred vitamin nomenclature in the United States and the Committee's efforts are being redirected to other problems in nutritional nomenclature and terminology. Close liaison with the IUNS Committee on Nomenclature and other official bodies is being maintained.

VITAMINS AND RELATED COMPOUNDS

Tentative rules for generic descriptors and trivial names

The AIN Committee on Nomenclature has undertaken to set forth the following Tentative Rules for Vitamins and Related Compounds following customary usage in the United States. A Third Draft of these Tentative Rules dated May 13, 1968 was widely circulated. The Tentative Rules as set forth in this Fourth Draft dated February 28, 1969 incorporates several changes suggested by the IUPAC-IUB Commission on Nomenclature, by Professor H. Dam and Dr. T. Moore representing the IUNS Committee on Nomenclature and by representatives of the Editorial Board, Journal of Nutrition. In addition, they are consistent with the nomenclature used by the Food and Nutrition Board, NAS-NRC in their Seventh Edition, 1968, of "Recommended Dietary Allowances."

In many instances, the various IUPAC-IUB-CBN "Tentative Rules" suggest alternate names for compounds with vitamin activity. This Committee feels that synonyms for a specific chemical compound are undesirable and lead to unnecessary complications in indexing and in computer retrieval. Therefore, only one name is suggested for each vitamin or vitamin-like compound.

Trivial names and generic descriptors for the vitamins and related compounds are useful and necessary in nutrition literature. IUPAC-IUB-CBN has advised this Committee that it considers the problem of generic descriptors of vitamins as lying outside its province and within the province of nutritionists.

In each instance, the generic descriptor should be used to describe the class of compounds having vitamin activity and to modify such terms as "activity," "deficiency," etc. The trivial names should be used only to identify the specific compound.

Fat-soluble vitamins and related compounds

V.1 Vitamin A

1.1 The term *vitamin A* should be used as the generic descriptor for all β-ionone derivatives except provitamin A carotenoids exhibiting qualitatively the biological activity of retinol. Thus, phrases such as "vitamin A activity" and "vitamin A deficiency" represent preferred usage.

1.2 The compound also known as vitamin A, vitamin A alcohol, vitamin A_1, vitamin A_1 alcohol, axerophthol or axerol should be designated *retinol*.

1.3 The compound also known as vitamin A (A_1) aldehyde, retinene or retinal should be designated *retinaldehyde*.

1.4 The compound also known as vitamin A acid should be designated *retinoic acid*.

1.5 The compound also known as vitamin A_2 or 3-dehydroretinol should be designated *dehydroretinol*.

1.6 The compound also known as retinene-2, 3-dehydroretinal, 3-dehydroretinaldehyde, or dehydroretinal should be designated *dehydroretinaldehyde*.

1.7 The compound also known as 3-dehydroretinoic acid should be designated *dehydroretinoic acid*.

V.2 Provitamin A Carotenoids

2.1 The term *provitamin A carotenoid* should be used as the generic descriptor for all

carotenoids exhibiting qualitatively the biological activity of β-carotene. When referring to the biological activity of the provitamin A carotenoids, the phrases "provitamin A activity" or "retinol equivalence" represent preferred usage.

2.2 Trivial names for specific carotenoids are under consideration.

V.3 Vitamin D

3.1 The term *vitamin D* should be used as the generic descriptor for all steroids exhibiting qualitatively the biological activity of cholecalciferol. Thus, phrases such as "vitamin D activity" and "vitamin D deficiency" represent preferred usage.

3.2 The compound also known as vitamin D_2 or calciferol should be designated *ergocalciferol.*

3.3 The compound also known as vitamin D_3 should be designated *cholecalciferol.*

V.4 Vitamin E

4.1 The term *vitamin E* should be used as the generic descriptor for all tocol derivatives exhibiting qualitatively the biological activity of α-tocopherol. Thus, phrases such as "vitamin E activity" and "vitamin E deficiency" represent preferred usage.

4.2 The term *tocopherols* should be used as the generic descriptor for all methyl tocols. Thus, the term "tocopherol" is not synonymous with the term "vitamin E."

4.3 The compound isolated from natural sources also known as 2-D, 4′D, 8′D-α-tocopherol, 2-R, 4′R, 8′R-α-tocopherol or *d*-α-tocopherol should be designated *α-tocopherol.*

4.4 The mixture of the eight possible stereoisomers of α-tocopherol commonly synthesized from racemic isophytol and also known as 2-DL, 4′DL, 8′DL-α-tocopherol, 2-RS, 4′RS, 8′RS-α-tocopherol or *dl*-α-tocopherol should be designated as *all-rac.-α-tocopherol.*

4.5 Trivial names for the stereoisomers of α-tocopherol are under consideration.

4.6 The compound also known as 5,8-dimethyltocol should be designated *β-tocopherol.*

4.7 The compound also known as 7,8-dimethyltocol should be designated *γ-tocopherol.*

4.8 The compound also known as 8-methyltocol should be designated *δ-tocopherol.*

4.9 The compound also known as ζ_1 or ζ_2-tocopherol, 5,7,8-trimethyltocotrienol, or tocochromanol-3 should be designated *α-tocotrienol.*

4.10 The compound also known as ε-tocopherol or 5,8-dimethyltocotrienol should be designated *β-tocotrienol.*

4.11 The compound also known as η-tocopherol, 7,8-dimethyltocotrienol, or plastochromanol-3 should be designated *γ-tocotrienol.*

4.12 The compound also known as 8-methyltocotrienol should be designated *δ-tocotrienol.*

V.5 Vitamin K

5.1 The term *vitamin K* should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone and all derivatives exhibiting qualitatively the biological activity of phylloquinone. Thus, phrases such as "vitamin K activity" and "vitamin K deficiency" represent preferred usage.

5.2 The compound 2-methyl-3-phytyl-1,4-naphthoquinone also known as vitamin K_1 should be designated *phylloquinone.*

5.3 The compound also known as vitamin K_2 should be designated as *menaquinone*-6.

5.4 The compound 2-methyl-1,4-naphthoquinone also known as menadione should be designated *menaquinone.*

5.5 Related compounds with vitamin K activity are described in the IUPAC-IUB Tentative Rules for "Nomenclature of Quinones with Isoprenoid Side Chains." (J. Biol. Chem., *241:* 2989–2991 (1966)). Preference is expressed for Proposal I in these recommendations.

Water-Soluble Vitamins and Related Compounds

V.6 Folacin

6.1 The term folacin should be used as a generic descriptor for all folates exhibiting qualitatively the biological activity of tetrahydropteroylglutamic acid. Thus, phrases such as "folacin activity" and "folacin deficiency" represent preferred usage.

6.2 The term *folate* should be used as the generic descriptor for the family of compounds containing the pteroic acid nucleus. Thus, the term "folate" is not synonymous with the term "folacin."

6.3 The compound also known as $PGAH_4$, THFA, tetrahydrofolacin or tetrahydrofolic acid should be designated *tetrahydropteroylglutamic acid* or abbreviated to H_4PteGlu.

6.4 The compound also known as N^5-F-$PGAH_4$, citrovorum factor, "CF," leucovorin, folinic acid, N^5-formyl THFA or N^5-formyltetrahydropteroylglutamic acid should be designated as *5-formyltetrahydropteroylglutamic acid* or abbreviated to 5-CHO-H_4PteGlu.

6.5 The compound also known as N^{10}-F-$PGAH_4$, heat-labile citrovorum factor, "HLCF", N^{10}-formyl THFA or N^{10}-formyltetrahydropteroylglutamic acid should be designated as *10-formyltetrahydropteroylglutamic acid* or abbreviated to 10-CHO-H_4PteGlu.

6.6 The compound also known as N^5-M-$PGAH_4$, "pre-folic A," N^5-methyltetrahydrofolacin, N^5-methyl THFA or N^5-methyltetrahydropteroylglutamic acid should be designated *5-methyltetrahydropteroylglutamic acid* or abbreviated to 5-CH_3H_4PteGlu.

6.7 Related compounds with folacin activity should be named and abbreviated in accordance with the IUPAC-IUB Tentative Rules "Nomenclature and Symbols for

Folic Acid and Related Compounds" (J. Biol. Chem., *241:* 2991–2992 (1966)).

V.7 Niacin

7.1 The term *niacin* should be used as the generic descriptor for pyridine 3-carboxylic acid derivatives exhibiting qualitatively the biological activity of nicotinic acid. Thus, phrases such as "niacin activity" and "niacin deficiency" represent preferred usage.

7.2 The compound, pyridine 3-carboxylic acid, also known as niacin, should be designated *nicotinic acid.*

7.3 The compound also known as niacinamide or nicotinic acid amide should be designated *nicotinamide.*

V.8 Riboflavin

8.1 The compound also known as vitamin B_2, lactoflavin(e) or riboflavine, should be designated *riboflavin.* Its use as a generic descriptor in such phrases as "riboflavin activity" and "riboflavin deficiency" represents acceptable usage.

V.9 Thiamin

9.1 The compound also known as vitamin B_1, aneurin(e) or thiamine should be designated as *thiamin.* Its usage as a generic descriptor in such phrases as "thiamin activity" and "thiamin deficiency" represents acceptable usage.

V.10 Vitamin B_6

10.1 The term *vitamin B_6* should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine. Thus, phrases such as "vitamin B_6 activity" and "vitamin B_6 deficiency" represent preferred usage.

10.2 The compound 3-hydroxy-4,5-bis(hydroxymethyl)-2-methylpyridine also known as vitamin B_6, adermin or pyridoxol should be designated *pyridoxine.*

10.3 The compound also known as pyridoxaldehyde should be designated *pyridoxal.*

10.4 The compound 3-hydroxy-4-methylamino-5-hydroxymethyl-2-methylpyridine should be designated *pyridoxamine.*

V.11 Vitamin B_{12}

11.1 The term *vitamin B_{12}* should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. Thus, phrases such as "vitamin B_{12} activity" and "vitamin B_{12} deficiency" represent preferred usage.

11.2 The term *corrinoids* should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term "corrinoid" is not synonymous with the term "vitamin B_{12}."

11.3 The compound α-(5,6-dimethylbenzimidazolyl)cobamide cyanide also known as vitamin B_{12} or cyanocobalamine should be designated *cyanocobalamin.*

11.4 The compound α-(5,6-dimethylbenzimidazolyl)hydroxocobamide also known as vitamin B_{12a}, vitamin B_{12b}, aquocobalamin or hydroxocobalamine should be designated *hydroxocobalamin.*

11.5 The compound α-(5,6-dimethylbenzimidazolyl)cobamide nitrite also known as vitamin B_{12c} or nitritocobalamine should be designated *nitritocobalamin.*

11.6 Related compounds with vitamin B_{12} activity should be named in accordance with the IUPAC-IUB Tentative Rules "Nomenclature of Corrinoids" (J. Biol. Chem., *241:* 2992–2994 (1966)).

V.12 Vitamin C

12.1 The term *vitamin C* should be used as the generic descriptor for all compounds exhibiting qualitatively the biological activity of ascorbic acid. Thus phrases such as "vitamin C activity" and "vitamin C deficiency" represent preferred usage.

12.2 The compound also known as vitamin C or L-ascorbic acid should be designated *ascorbic acid.*

12.3 The compound also known as L-dehydroascorbic acid should be designated *dehydroascorbic acid.*

V.13 Pantothenic Acid

13.1 The compound also known as pantoyl-β-alanine should be designated *pantothenic acid.* Use of this term as a generic descriptor in such phrases as "pantothenic acid deficiency" represents acceptable usage.

V.14 Biotin

14.1 The compound also known as "coenzyme R" should be designated as *biotin.* Use of this term as a generic descriptor in such phrases as "biotin activity" and "biotin deficiency" represents acceptable usage.

V.15 Choline

15.1 The compound choline should be designated *choline.* Use of this term as a generic descriptor in such phrases as "choline activity" and "choline deficiency" represents acceptable usage.

V.16 Myo-inositol

16.1 The compound also known as inositol or mesoinositol should be designated *myo-inositol.* Use of this term as a generic descriptor in such phrases as "myo-inositol activity" and "myo-inositol deficiency" represents acceptable usage (see European J. Biochem., *5:* 1–12 (1968)).

V.17 p-Aminobenzoic Acid

17.1 The compound p-aminobenzoic acid should be designated *p-aminobenzoic acid.* Use of the term as a generic descriptor in such phrases as "p-aminobenzoic acid activity" and "p-aminobenzoic acid deficiency" represents acceptable usage.

ABBREVIATED DESIGNATION OF AMINO ACIDS

The AIN Committee on Nomenclature has reviewed the Rules proposed by the IUPAC-IUB Commission on Biochemical Nomenclature for designation of amino acids and peptides. The committee sees no conflict or any special problems with the naming of amino acids in nutrition. The committee, therefore, recommends that we accept the Rules as they were presented (1, 2, 3) by the IUPAC-IUB Commission on Biochemical Nomenclature and that when new additions or modifications are accepted by their commission they be automatically accepted as a guide for the AIN.

Since the naming and sequencing of amino acids in natural or synthetic peptides is not commonly found in the nutritional literature, no repetition of these rules seems necessary; the reader is simply referred to the original description of the Tentative Rules (2, 3). Since amino acids are routinely referred to in nutrition with particular needs for abbreviations, the following is reprinted here for easy reference for the nutritionist. (Taken from 1.)

1. *General considerations*

1.1 The symbols chosen are derived from the trivial names or chemical names of the amino acids and of chemicals reacting with amino acids and polypeptides. For the sake of clarity, brevity, and listing in tables, the symbols have been, wherever possible, restricted to three letters, usually the first letters of the trivial names.

1.2 The symbols represent not only the names of the compounds but also their structural formulas.

1.3 The amino acid symbols by themselves represent the amino acids. The use of the symbols to represent the free amino acids is *not* recommended in textual material, but such use may occasionally be desirable in tables, diagrams, or figures. Residues of amino acids are represented by addition of hyphens in specific positions as indicated in Section 3.

1.4 Heteroatoms of amino acid residues (e.g. O^{β} and S^{β} of serine and cysteine, respectively, N^{ϵ} of lysine, N^{α} of glycine, etc.) do not explicitly appear in the symbol; such features are understood to be encompassed by the abbreviation.

1.5 Amino acid symbols denote the L configuration unless otherwise indicated by D or DL appearing before the symbol and separated from it by a hyphen. When it is desired to make the number of amino acid residues appear in a clearer manner, the hyphen between the configurational prefix and the symbol may be omitted (see Ref. 1). (Note: The designation of an amino acid residue as DL is inappropriate for compounds having another amino acid residue with an asymmetrical center.)

1.6 Structural formulas of complicated features may be used along with the abbreviated notation wherever necessary for clarity.

2. *Abbreviations for amino acids*

2.1 *Common Amino Acids*

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine [5]	Asn [5]	Methionine	Met
Aspartic acid	Asp	Ornithine	Orn
Cysteine	Cys	Phenylalanine	Phe
Cystine	Cys-Cys *	Proline	Pro
Glutamic acid	Glu	Serine	Ser
Glutamine [5]	Gln [5]	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

[5] Asparagine and glutamine may also be denoted as Asp (NH_2) or Asp, and Glu (NH_2) or Glu, respectively.
(with NH_2 attached below Asp and Glu by a vertical bond)

* Cystine does not appear in 2.1 (ref. 1). We propose Cys-Cys as consistent with 2.1 but this abbreviation should not be used to denote structure.

2.2 *Less Common Amino Acids* — Abbreviations for less common amino acids should be defined in each publication in which they appear. The following principles and notations are recommended.

2.2.1 Hydroxyamino Acids

Hydroxylysine	Hyl
3-Hydroxyproline	3Hyp
4-Hydroxyproline	4Hyp

2.2.2 *allo*-Amino Acids

allo-Isoleucine	aIle
allo-Hydroxylysine	aHyl

2.2.3 *"Nor" Amino Acids* — "Nor" (*e.g. in norvaline*) is not used in its accepted sense (denoting a lower homologue) but to change the trivial name of a branched chain compound into that of a straight chain compound (compare with "iso," paragraph 2.1). "Nor" should therefore be treated as part of the trivial name without special emphasis.

Norvaline	Nva
Norleucine	Nle

2.2.4 *Higher Unbranched Amino Acids* — We suggest the following general rules for guidance in forming abbreviations: the functional prefix "amino" should be included in the symbol as the letter "A," diamino as "D."

The trivial name of the parent acid should be abbreviated to leave no more than two or three letters, as convenient and necessary for clarity. The word "acid" ("*-saure,*" etc.) should be omitted from the symbol as carrying no significant information. Unless otherwise indicated (see Ref. 1), single groups are in the α position, two amino groups in the α,ω (monocarboxylic acids) or α,α' positions (dicarboxylic acids). The location of amino acids in positions other than α and ω is shown by the appropriate Greek letter prefix.

Examples:

α-Aminobutyric acid	Abu
α-Aminoadipic acid	Aad
α-Aminopimelic acid	Apm
α,γ-Diaminobutyric acid	Dbu
α,β-Diaminopropionic acid	Dpr
α,α′-Diaminopimelic acid	Dpm
β-Alanine	βAla
ε-Aminocaproic acid	εAcp
β-Aminoadipic acid	βAad

For further details in using symbols to designate structure, the reader is referred to the following references (in order: 1, 2, 3).

REFERENCES

1. IUPAC-IUB Commission on Biochemical Nomenclature 1966 Abbreviated designation of amino acid derivatives and peptides. Tentative Rules, J. Biol. Chem., *241*: 2491–2495.
2. IUPAC-IUB Commission on Biochemical Nomenclature 1967 Rules for naming synthetic modifications of natural peptides. Tentative Rules, J. Biol. Chem., *242*: 555–557.
3. IUPAC-IUB Combined Commission on Biochemical Nomenclature 1968 Tentative rules for abbreviated nomenclature of synthetic polypeptides (polymerized amino acids). Biochemistry, *7*: 483–485.

E. *AIN Representative to the Food and Agriculture Organization*: H. E. Sauberlich.

The following information is provided concerning FAO and WHO activities of interest to nutritionists:

1. *Nutrition Newsletter.* This publication is published quarterly by the Nutrition Division of FAO. The publication provides a concise report of FAO activities, including special nutrition articles, list of FAO recent publications, reports on FAO sponsored meetings and seminars, outside nutrition meetings, etc. The publication should be followed by all nutritionists, and particularly those with international interests. Information concerning subscriptions should be addressed to: The Editor, Nutrition Newsletter, Nutrition Division, FAO of the United Nations, Via delle Terme di Caracalla, 00100 Rome, Italy.

2. *Joint FAO/WHO/OAU(STRC) Regional Food and Nutrition Commission for Africa.* The commission periodically publishes special papers that should be of interest to most nutritionists, although with emphasis on nutrition problems of Africa. The following are examples:

A. May, 1968: Special Paper No. 3, "Nutrition and Working Efficiency (with special reference to the tropics)," by F. W. Lowenstein, WHO Secretary.

B. July, 1968: Special Paper No. 4 "A Survey on Nutrition Research in Tropical Africa," by The Secretariat.

The articles may be obtained by writing to: Joint FAO/WHO/OAU(STRC) Regional Food and Nutrition Commission, P.O. Box 1628, Accra, Ghana.

3. *CAJANUS: Newsletter of the Caribbean Food and Nutrition Institute.* This institute is supported in part by FAO and WHO. The newsletter is in its second year of publication and contains various articles of nutrition interest, with emphasis on the problems of the Caribbean area. Copies of the publication may be obtained by writing to: The Caribbean Food and Nutrition Institute, Mona P. O. Box 140, Kingston 7, Jamaica.

4. A recent FAO publication is entitled "Food and Nutrition Procedures in Times of Disaster." A complete listing of all FAO or WHO publications is available in catalogues published by the respective organizations. In 1968, WHO published such a catalogue entitled "1947–1967 Catalogue of World Health Organization Publications." Copies of these publications catalogues may be obtained by writing to:

A. WHO Publications: Columbia University Press, 136 South Broadway, Irvington-on-Hudson, New York, New York 10533

B. FAO Publications: Columbia University Press, International Documents Service, 2960 Broadway, New York, New York 10027

F. *Public Affairs Committee:* H. A. Schneider, Chairman.

In 1967 the Federation established an Office of Public Affairs with Mr. Robert Grant as Director. Simultaneously, there was established a FASEB Public Affairs Committee made up of one member from each of the six constituent societies and three members-at-large. The AIN, at the same time, appointed a Public Affairs Committee, the chairman of which was to serve as AIN member of the FASEB Committee. The present members of the AIN Committee are H. A. Schneider, chairman, O. L. Kline and Leroy Voris. The functions of the FASEB Committee are to consider, discuss, and approve activities appropriate for FASEB participation, and to serve in an advisory capacity to the Office of Public Affairs. Significant actions and activities are described in a newsletter which issues frequently from Mr. Grant's office.

During the past year the Public Affairs Committee of FASEB met in September, January, and April. On these occasions there have been briefings by the Director of the National Institutes of Health and the Deputy Director of the Office of Science and Technology, the White House. Position papers were prepared on various bills before the Congress, and on issues affecting the scientific community. For example, the Committee debated the relative merits of the so-called Rogers-Javits Bill on revamping the present system of laboratory animal care, the regulation of research facilities, and dealer traffic in laboratory animals. The Committee decided not to endorse the bill for a variety of reasons, but most importantly that the present law was put into effect only two years ago and is still relatively untested (it has never been fully funded). A position paper was then prepared, adopted, and H. A. Schneider was delegated to represent the Federation and its position whenever Congressional hearings are held.

In a similar manner the Public Affairs Committee and the Office of Public Affairs has responded to issues such as the drafting of graduate students, the institutional support of the education of scientists by the National Science

Foundation, and the appearance of two Federation witnesses, Drs. E. H. Lennette and G. M. Briggs, before the House Subcommittee on Appropriations in support of the NIH budget and of biomedical research.

Recently, on the initiative of the AIN Public Affairs Committee and the AIN Council, the FASEB Public Affairs Committee supported the creation of a national program capable of responding to the disclosure of hunger and malnutrition in the United States. The position paper on this subject, encouraging appropriate legislative action, approved by the Committee and forwarded to Senator Yarborough, Chairman, Subcommittee on Health Legislation, was as follows:

POSITION PAPER ON HUNGER AND MALNUTRITION IN THE UNITED STATES

The Federation of American Societies for Experimental Biology, through its Committee on Public Affairs, has been alerted by the American Institute of Nutrition, a constituent society of the Federation, to recent and continuing reports on the prevalence of hunger and malnutrition at unacceptable levels in this country. The Federation feels compelled to observe that the application and delivery of scientific knowledge, in this instance, has fallen below reasonable estimates of what is desirable and possible at the present stage of nutritional information and resources. The Federation is fully aware that the application of scientific knowledge must proceed within a framework of cultural and economic realities. However, the time is now overdue for a review of the fragmented and divided activities of the National Government which, unfortunately, are not now coherent enough to conceive and deliver an adequate capability for coping with hunger and malnutrition.

To achieve this mission the Federation urges the adoption of the following principles:

1. That existing field, laboratory and educational efforts should be consolidated and coordinated, for efficiency, into a single visible program of nutrition for the Nation.

2. That a continuing mechanism should be assembled to monitor the nutritional health of the American people with a view to the early identification and prevention of pockets of hunger and malnutrition: a Hunger Watch.

3. That priorities be assigned to the implementation of a system of nutritional health delivery, utilizing all resources, so that the abundance of the American land be made available to all of its citizens.

With these principles in mind, the Federation stresses that immediate action by the Federal Government is now imperative. No less than the health of some of our fellow citizens is at stake, and needlessly so, for the knowledge and capacity to erase this blot exists right now. We call upon the Congress to enact the required legislation, to do what we all think we *must* do, so that there be forged a clear and single policy, a single program, and so that hunger and malnutrition be truly banished from the United States.

G. *AIN Representative to the American Association for the Advancement of Science:* H. C. Tidwell.

AAAS Council Meeting, Dallas, 1968. A meeting of AAAS and the British Association for Advancement of Science was planned to consider activities and responsibilities of general science societies devoted to an improvement of public understanding of science and to advancing scientific research and study.

The Council recommended and the Board of Directors approved changes in statements regarding planned studies on war time use of herbicides in Vietnam. The Council wished to avoid specific mention of political, as distinct from scientific, organizations and to avoid any implication that the Association was criticizing the Government of the United States. It was stressed that contributions to the study on the war and peace time use of herbicides from all available sources should be accepted.

The Committee on Council Affairs met with government representatives to discuss fuller and more consideration of the balance among the criteria that should be considered in reaching decision on future large-scale construction projects that may affect the national or regional economy. The loss of valuable agricultural land by such constructions has been referred to the Committee on Environmental Alteration for further study.

The Council Affairs Committee has recommended and the Board agreed to appoint a special committee to consider whether the criteria for affiliation should be clarified and perhaps changed. Also whether affiliation should be withdrawn from an affiliate who has violated the criteria for affiliation or given evidence of prolonged lack of interest in AAAS affairs.

The desirability of a restructuring of the Council so as to strengthen the Association in carrying out some of its responsibilities was discussed. Four areas suggested were: 1) A more general participation of scientists in the government of science and science in the aid of government, 2) the promotion of the integrity of science, 3) social responsibility of science, and 4) in advancing a greater public understanding of science. The Council voted that the matter of reorganization be further considered and a poll of the Council on questions involved, be made for a report to the 1969 meeting.

Additional details may be obtained by referring to Science, *163:* 832 (1969).

H. *Symposia Committee:* G. F. Combs.

Council approved the proposed symposia topics as presented by Dr. Combs for the 1970 program.

Topics are: Energy costs of intermediate metabolism in the intact animal; Trace elements on metabolism of connective tissue; Biochemical indicators of nutritional status; and The history of nutrition.

The history of nutrition is scheduled as the first symposium to be supported in part by the Wendell H. Griffith Memorial.

I. *AIN Representative to the National Research Council Boards:* G. F. Combs.

Dr. Philip Handler, Chairman of the Department of Biochemistry, Duke University Medical Center, succeeds Dr. Fredrick Seitz as President of The National Academy of Science. Dr. Donald S. Farner, Department of Zoology, University of Washington, assumes the post of Chairman, Division of Biology and Agriculture, from Dr. A. Geoffrey Norman. The Division of Biology and Agriculture celebrates its 50th Anniversary, having been established in April 1919.

The Food and Nutrition Board was reorganized to provide for rotation of its members with Dr. Mark Hegsted, succeeding Dr. Grace Goldsmith as Chairman. Dr. William J. Darby was appointed Vice Chairman. The following board members were reappointed with the expiration dates listed in parentheses:

D. L. Call, (1971); G. K. Davis, (1969); Grace Goldsmith, (1969); D. B. Hand, (1970); A. E. Harper, (1971); H. E. O. Heineman, (1969); L. M. Henderson, (1971); J. F. Mueller, (1970); B. S. Schweigert, (1971); N. S. Scrimshaw, (1971); W. H. Sebrell, Jr., (1970); R. E. Shank, (1970).

The Food and Nutrition Board was asked to give particular attention to (1) the world food problem; (2) nutrition problems in the United States; and (3) the training of physicians, nutritionists and other health personnel in nutrition.

Dr. Allen Forbes, Army Research Office, succeeds Dr. O. L. Kline as Chairman of The U. S. National Committee of The International Union of Nutrition Sciences.

New NRC publications relating to nutrition include:

1694 *Recommended Dietary Allowances* (1968 Revision)
1599 *Nutrient Requirements of Swine* (1968 Revision)
1676 *Nutrient Requirements of Mink and Foxes* (1968 Revision)
1693 *Nutrient Requirements of Sheep* (1968 Revision)
1684 *U. S.-Canadian Tables of Feed Composition* (1968 Revision)
1598 *Body Composition of Animals and Man* — Proceedings of a Symposium (1969)
1679 *The Use of Drugs in Animal Feeds* — Proceedings of Medicated Feeds Symposium (1969)

J. *AIN Office of Nutrition Science Services:* O. L. Kline, Director.

1. *Final Report on the Project to Provide Scientific Rosters and Related Informational Services for the Agency for International Development (AID).* This project which has been in progress since December 1, 1966 terminated on November 15, 1968, a period of nearly two years to its completion. The purpose of the project as outlined in the contract was to provide to the Project Officer, Public Health Service, assistance and informational services in the development of nutrition programs in pilot countries supported by AID. Some of these requirements are summarized below; a complete final report is on file in the AIN office.

Two rosters have been collected: a roster of over 500 persons highly trained in experimental and pediatric nutrition who might be available to accept assignments in pilot countries' nutrition programs, or in the development of U. S. effort in foreign assistance and a roster of more than 2700 names of foreign students trained in the U. S., either at the B.S. or graduate level. Thirty-three libraries and institutions in developing countries were identified and offered complimentary subscriptions to nutrition journals. Fourteen of these have been supplied with journal subscriptions of their choice; the remainder did not reply to the invitation.

The Office undertook the preparation of the U. S. Government reply to the United Nations General Assembly questionnaire on the U. S. efforts to increase and improve the world protein supply. Funds were provided for the support of consultants who assisted in collecting the information requested and in the coordination and preparation of the report.

Information was collected from food technologists and nutritionists in 15 countries, mostly developing countries, and from a number of food manufacturers in the U. S. as to activities in developing new foods high in protein, directed to the problems of child feeding.

It has been estimated that from 25 to 50% of the food produced in some less-developed countries is lost through rodent and insect infestation, spoilage in storage, and in transportation and losses in the market place. The office played an active role in proposing and establishing a course of training for food inspectors in the University of Puerto Rico School of Public Health. This development is sponsored by the Pan American Health Organization.

2. *Activities of the IUNS.* The U. S. National Committee for IUNS has been active since 1955 and has had as one of its principal objectives the admission of the IUNS into the International Council of Scientific Unions (ICSU). ICSU is a federation of 16 member unions, representing the basic and physical and biological sciences, supported by the United Nations. It is through ICSU

that broad international programs in science are initiated and sponsored.

The IUNS was admitted to ICSU by an overwhelmingly favorable vote in September 1968. Membership in ICSU will increase greatly the opportunity for cooperation with other member Unions, and will improve our capability to deal with some of the most crucial social and scientific problems facing the world today.

A pre-Congress meeting of commissions and committees of the IUNS will be held in Yugoslavia August 20–23, 1969. There has been some discussion on the establishment of an international committee on congresses to lighten the administrative functions of local committees.

3. The AIN Office of Nutrition Science Services is presently under a temporary contract with NIH for the development of a continuing source of nutrition information from appropriate United Nations agencies.

4. Application has been filed with the National Science Foundation for funds for staff support for the Nutrition Program of the International Biological Program. When funded, this would be the third major project undertaken by this Office since its establishment in late 1966.

K. *Report on the International Biological Program:* G. K. Davis.

Nutrition is classified as an intersectional program for the International Biological Program (IBP) and is jointly between the Section on Use and Management and the Section on Human Adaptability. An evaluation of the nutrition program is that it continues to contribute solidly to the programs on human adaptability and is a vital part of the project on circumpolar peoples and the program on high altitude adaptation and is part of the different biomedical studies of grasslands and woodlands.

At the international level and in part as a result of suggestions from the U. S. group it has been proposed that the ecological aspects of nutrition might be given particular interest in the evaluation of nutrition as related to parasitism since this is an almost universal ecological factor in the nutrition of most humans and animals. The question is asked, what is the relationship between parasitic infection and nutrition requirements and the efficiency of nutrition utilization? The second aspect with an ecological role is the relationship between genetics and nutrient requirement and seriously being considered is the possibility of mounting a nutrition program internationally to evaluate the response of individuals with differing genetic background to nutrition and the influence of the environment on such response.

There is no particular objection to the various projects which have been included with special emphasis on the relationship between nutrition and physical and mental development, the relationship between nutrition and the development of disease and immunity to disease, on the development of nutrition standards for various different environmental conditions with its corollary the evaluation of the applicability of standards developed by the U.S., the U.K., and F.A.O. to the various environmental situations, especially the tropics, but there has been a strong feeling that there is not a central rallying point for nutrition as a major push. Contribution to the International Biological Program would be increased with well developed integrated nutrition studies.

There is a desire on the part of many who would like to give much stronger support to nutrition for the identification of an ecological basis for a nutrition program. It is recognized that many, if not all, of the nutrition projects included in IBP have elements of this in them. But, it is not the overriding or coordinating theme that it might be.

From the standpoint of IBP as an international project a principal limitation has been funding. There is a strong move to have the IBP provide the scientific basis for the intergovernmental programs scheduled for 1971, 1972 and beyond, which has been titled, "Man and the Biosphere."

Nationally, the IBP has received very favorable hearings in the Congress. Bills have been introduced and hearings held which give very strong encouragement to the idea that specific funds will be appropriately earmarked for the IBP (through the National Science Foundation). At present the division of funds which has been suggested indicates that some sixty percent might be allocated to the different ecological studies of the biomes and forty percent allocated to studies including human adaptability and the use and management areas which include nutrition. The actual implementation of this depends, of course, upon the action of the Congress and the appropriation bills have a way of being rather late in their passage. In the U. S. we have established an integrated program in nutrition which has a strong ecological base.

Generally speaking, the IBP has begun to catch on around the world with some fifty countries with active national programs and national committees for the IBP and some eighty countries with projects of varying degrees of sophistication operational under the IBP.

L. *Report of the AIN Archivist:* E. Neige Todhunter.

AIN Archivist is interested in bringing together the materials that relate to the history of nutrition in the American Institute of Nutrition and in preserving the materials on the AIN in a manner that would make it possible to continue the history of the AIN. Council has authorized the continuation of publication of biographical sketches in the Journal of Nutrition and has en-

couraged the continued exploration of ways and means for preserving the voices in interview of historical nutritionists and through video tape.

M. *Guide to Nutrition Terminology:* E. Neige Todhunter.

Nutrition science and all the applications of nutrition in the feeding of people provide an extensive and ever-growing list of nutrition publications. It is becoming increasingly difficult to store this literature in an appropriate place, and even more difficult to be able to retrieve what is needed at any given moment on a given topic.

This problem is not unique to nutrition. It has been estimated that about 1.5 million scientific documents should be indexed each year. Projecting into the future, Mr. J. Sherrod of the Library of Congress estimates that about year 2000 one million technical journals will be in publication. Specialists in a number of areas are actively working on this problem. One method currently being used is the compilation of a thesaurus or comprehensive list of terms for indexing, storing, and systematically retrieving information relevant to a specific subject matter field. Several have been developed and are currently undergoing usage tests.

There is a definite need for a Guide to Nutrition Terminology applicable to the broad field of modern nutrition. Such a Guide is now in preparation at Vanderbilt University under a contract supported by the National Institute of Arthritis and Metabolic Diseases and the National Institute of Child Health and Human Development. The principal investigator is Dr. E. Neige Todhunter who is working with guidance from an advisory committee of scientists representing clinical nutrition, animal nutrition, biochemistry, pediatrics, and the behavioral sciences. Liaison members from AIN, FAO, WHO, Food and Nutrition Board of the NAS/NRC, National Library of Medicine, and National Agricultural Library (USDA) also meet with the advisory committee and Dr. Todhunter.

The Guide to Nutrition Terminology is being prepared in three parts. There will be 1) a listing of terms consisting of twelve categories with approximately seventy subcategories and hierarchical arrangement of terms within subcategories; 2) an alphabetical listing of all terms with synonyms and related terms shown wherever appropriate and helpful as a guide to the user; 3) a glossary for all the terms which need some clarification of indication of the way in which they are used in the terminology guide.

Terms being selected for inclusion in the vocabulary are those appearing with some frequency in the current nutrition literature. However, the term listings are not planned nor intended to be complete and exhaustive. Rather, the compilation is intended as a guide to or organizational framework for nutrition terminology. Additional terms or new terms can readily be inserted into the listings at any point where a specialist in a given area may want greater depth or more specific terms for his own use. The Guide will be in a form in which it can be coded to meet individual needs, or adapted to the systems of manual, machine, or computerized operation being used by any given organization.

The Guide is planned to be of assistance not only to libraries but to members of AIN and other groups or individuals who are concerned with storage and retrieval of information on various aspects of nutrition. A special effort will be made to incorporate appropriate portions of the Guide into major existing literature storage and retrieval facilities. As an example, NICHD plans to utilize the Guide terminology in the retrieval system being established for its Information Centers program. Although not specifically included in current contract activities, it is possible that this Guide might form the basis for a recurring bibliography to nutrition literature, much as has been developed from similar projects in endocrinology and rheumatology.

Present plans call for completion of the Guide in 1969 with preliminary copies then going to interested nutrition scientists for evaluation and testing as to how effective it is in meeting their needs.

XII. *Votes of Thanks*

Sincere appreciation and thanks were expressed also for the service of all outgoing committee chairmen and members, and the outgoing councilor, Dr. A. E. Harper. A standing ovation was given to outgoing President Richard H. Barnes.

XIII. *Future Annual Meetings*

1970: April 12–17, Atlantic City, N. J.

1971: April 12–17, Chicago, Ill.

[Note: The American Society of Biological Chemists will meet in San Francisco, California, June 13–18, 1971.]

1972: April 9–14, Atlantic City, N. J.

1973: April 15–20, Atlantic City, N. J.

ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on Wednesday, April 16, 1969 at the Shelburne Hotel with 406 attending. Dr. Barnes presided.

Dr. R. M. Forbes, Chairman of the 1969 Fellows Committee, introduced the newly appointed Fellows, whose citations follow:

KARL E. MASON

Karl E. Mason — for a distinguished career in teaching, research and administration in the fields of nutrition, anatomy and physiology; for his early studies on nutritional factors in reproduction which led to his classic publications on the effects of vitamin A and E deficiency on the histology of the rat testis; for his subsequent work leading to discovery of the relatively poor vitamin E status of the newborn infant; for his continuing work on the importance of cadmium and other inorganic elements on testicular function; for his teaching career, highlighted by dealing with anatomy from the viewpoint of dynamic physiology with nutrition emphasis; for his service as Nutrition Program Director, National Institute of Arthritis and Metabolic Diseases. He is a past-president of the American Association of Anatomists.

Stanislaw K. Kon — for his outstanding contributions in many areas of nutritional sciences, especially in the field of fat-soluble vitamins, nutritive value of milk, protein evaluation and application of the science of nutrition in developing countries; for his contributions as Head of the Department of Nutrition and as Deputy Director of the National Institute of Research in Dairying, Shinfield, Reading, England; for his long career as the Editor-in-Chief of the *British Journal of Nutrition* and the *Proceedings of the Nutrition Society*. Among his many achievements, he was one of the first to recognize the importance of vitamin A_1 aldehyde in the visual process. He has been the recipient of many honors for his services to nutrition in the United Kingdom.

STANISLAW K. KON

BERNARD L. OSER

Bernard L. Oser — for an outstanding career in research, education and administration in the fields of nutrition and agricultural and food chemistry; for his many contributions in the areas of safety evaluation of food additives, pesticides and drugs, including methodology for biochemical and toxicological procedures; for his leadership in establishment of a uniform policy with respect to food additives at both the national and international level; for his contribution to education as lecturer in public health nutrition and as editor of *Hawk's Physiological Chemistry* and of the Food Drug Cosmetic Law Journal. For many years he has vigorously supported the areas of nutrition and food technology through active membership in numerous scientific societies and affiliations, including the Institute of Food Technology (President), American Institute of Nutrition, American Board of Nutrition.

AIN MEAD JOHNSON AWARD FOR RESEARCH IN NUTRITION

ROBERT H. WASSERMAN

The 1969 Mead Johnson Award of $1000 and a scroll was presented to Dr. Robert H. Wasserman, Professor of Radiation Biology, Department of Physical Biology, New York State Veterinary College, Cornell University. The award was given for his discovery, isolation, and elucidation of function of a specific protein found in homogenates of the intestine which binds calcium and requires vitamin D for its synthesis; and for the importance of these findings in enlarging our understanding of calcium metabolism and the functions of vitamin D.

BORDEN AWARD IN NUTRITION

HARRY P. BROQUIST

The American Institute of Nutrition's 1969 Borden Award of $1000 and a gold medal was presented to Dr. Harry P. Broquist, Professor of Biochemistry, University of Illinois. The award was given for the isolation and identification of a toxic agent in certain legume forages which causes excess salivation in dairy cattle; the discovery that citrovorum factor reverses the toxicity of aminopterin-type folic acid derivatives leading to the clinical use of this compound; the use of formimino glutamic acid as a measure of folic acid deficiency; and studies on the biosynthesis of lysine.

OSBORNE AND MENDEL AWARD

HAMISH N. MUNRO

The 1969 Osborne and Mendel Award of $1000 and a scroll was presented to Dr. Hamish N. Munro, General Foods Professor of Physiological Chemistry, Massachusetts Institute of Technology. The award was given for his outstanding basic and exploratory research in mammalian protein metabolism; for the effects of amino acids and other nutrients on tissue polysomes, on cellular synthesis of protein, and in the regulation of protein metabolism; and for demonstrating the influence of ribonucleic acid and iron on ferritin synthesis.

THE CONRAD A. ELVEHJEM AWARD FOR PUBLIC SERVICE IN NUTRITION

FREDERICK J. STARE

The 1969 Conrad A. Elvehjem Award of $1000 and a scroll was given to Dr. Frederick J. Stare, Professor and Chairman, Department of Nutrition, School of Public Health, Harvard University. The award was presented in recognition of his distinguished service as the articulate interpreter and advisor to a public increasingly interested in sound nutrition; the thoughtful editor of *Nutrition Reviews*; the wise advocate of nutritional research; and an indomitable champion of scientific truth in the public forum.

AMERICAN INSTITUTE OF NUTRITION

Founded September 27, 1928; Incorporated November 16, 1934; Member of Federation 1940

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Past President: R. H. Barnes, Graduate School of Nutrition, Cornell University, Ithaca, New York 14850.

Secretary: Doris H. Calloway, Department of Nutritional Sciences, University of California, Berkeley, California 94720 (1972).

Treasurer: H. W. Howard, Borden, Incorporated, 350 Madison Avenue, New York, N. Y. 10017, (1971).

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Committee on Publications Management: W. J. Darby (1970), chairman; J. F. Mueller (1970), F. L. Iber (1971), O. L. Kline (1970), James Waddell (continuing).

Nomenclature Committee: S. R. Ames (1970), chairman; E. W. Crampton (1970), Q. R. Rogers (1970), E. L. Hove (1972), G. A. Leveille (1972).

Archivist: E. N. Todhunter.

AIN Nutrition Notes, Editor: O. C. Johnson (1970).

Symposia Committee Chairman for 1970 Program: G. F. Combs.

Ad hoc Committee on Graduate Nutrition Training: A. E. Harper, chairman; W. D. Brown, Dena C. Cederquist, Hans Fisher, L. M. Henderson.

Ad hoc Committee on Undergraduate Nutrition Training: Olaf Mickelsen, chairman; Helen A. Hunscher, A. R. Kemmerer, Melvin Lee, Mary Ann Williams.

Ad hoc Committee for Nomination for National Medal of Science: R. E. Shank, chairman; B. C. Johnson, G. M. Briggs.

U. S. National Committee, International Union of Nutritional Sciences: A. L. Forbes (1971), chairman; W. A. Krehl (1970), A. E. Schaefer (1970), G. F. Stewart (1970), R. O. Nesheim (1971), R. E. Olson (1971), G. M. Briggs (1972), W. A. Gortner (1972), H. E. Sauberlich (1972). Ex officio (voting) B. L. O'Dell, D. M. Hegsted. Ex officio (non voting) C. G. King, A. G. Norman, R. K. Cannan, H. Brown, E. C. Rowan.

REPRESENTATIVES

American Association for the Advancement of Science: L. C. Fillios (1971), W. J. Monson (1971).

Food and Agriculture Organization: H. E. Sauberlich (1970).

National Society for Medical Research: P. H. Derse (1970).

National Research Council Boards and Divisions, Division of Biology and Agriculture, Agricultural Research Institute, and Food and Nutrition Board: J. G. Bieri.

Liaison U. S. National Committee for the International Biological Program: G. K. Davis.

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FASEB Executive Committee: B. L. O'Dell.

FASEB Long Range Planning Committee: W. J. Darby; H. W. Howard (alternate).

FASEB Program Committee: James Waddell.

FASEB Public Affairs Committee: H. A. Schneider.

FASEB Public Information Committee: M. S. Read.

Federation Proceedings Editorial Committee: Olaf Mickelsen.

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