Lactose and Calcium Transport in Gut Sacs[1]

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ABSTRACT The effects of glucose and lactose upon calcium absorption in rats were investigated using the inverted gut sac technique with Ca^{45}. In weanling animals calcium transport was stimulated by lactose both in the duodenum where calcium is absorbed against a concentration gradient and in the lower intestine where absorption is by passive diffusion. The ability of lactose to stimulate transport was lost as the animals became older, and was not clearly demonstrable after the animals were 6 weeks of age. The active transport of calcium against a concentration gradient in the upper intestine is partially dependent upon the amount of calcium in the diet the animals receive. Only a slight and insignificant effect of dietary calcium upon calcium transport in the lower intestine could be shown.

A considerable body of evidence relates lactose consumption to calcium absorption and metabolism. In Duncan's review in 1955, (1) she discusses papers related to the prevention of parathyroid tetany with lactose; the prevention of, or partial protection from, rickets by lactose; increases in bone mineral with lactose feeding; and the treatment of milk fever by udder inflation. The latter effect might in part be due to elevation of blood lactose. The explanation most often given for the effect upon calcium metabolism is that the lactose is slowly absorbed, resulting in a gastrointestinal flora which lowers the pH within the gut (1, 2). However, all studies are not consistent with this hypothesis (3). The formation of soluble non-ionized compounds or complexes has been proposed as a mechanism, the most recent evidence for their formation being that of Charley and Saltman (4). Others, especially Fournier (5–8) have postulated that lactose has a structural role and the effect upon calcium may be at the level of the bone cell.

In recent papers Lengemann et al. (9) confirmed the effect of lactose upon absorption utilizing the absorption of Ca^{45} from isolated gut loops in vivo. This effect was attributed to the slow absorption of lactose. Similarly, Vaughan and Filer (10) showed that many sugars promoted calcium absorption in the lower end of the gut. They concluded that the effect of lactose could be explained by slow absorption, thus providing effective sugars lower in the intestinal tract. No specific effect of lactose upon calcium absorption could be shown.

The recent studies of Schachter et al. (11, 12) and Harrison and Harrison (12) have shown that in the rat, calcium can be tranported against a concentration gradient only in the first few centimeters of the intestine. In most of the intestine the absorption of calcium is apparently by diffusion (13). The utilization of the everted gut sac technique of Wilson and Wiseman (14) provides more effective control of experimental conditions, albeit under somewhat less physiological conditions, than in vivo techniques. The data reported here, utilizing the everted gut sacs, apparently demonstrate an effect of lactose upon the passage of calcium through the wall of the intestinal tract. This effect could only be shown in quite young animals.

EXPERIMENTAL

Male weanling rats of the Holtzman strain were used and received either a low or high calcium diet. The low calcium diet was of the following percentage composition: casein, 18; glucose, 62.9; Ca-free salt mixture,[3] 2.5; hydrogenated cottonseed oil,[4] 9.5; cellulose,[5] 5: vitamin mixture,

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[3] Jones and Foster (14) salt mixture.
[4] Spry, Procter and Gamble Company, Cincinnati.
[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.

0.3[6]; cystine, 0.3; choline chloride, 0.3; and cod liver oil, 0.5. Calcium carbonate was added to the diet at a 0.2% level of calcium. The high calcium diet contained the same ingredients as that of group 1 except that calcium carbonate was added to the diet at a 1% level of calcium. The animals were placed in separate cages and water and food were supplied ad libitum when received and were studied at intervals thereafter. Food was removed from the cage the afternoon before the animals were decapitated.

In the studies on active transport, the first 3 to 4 cm of the intestine just distal to the pylorus were removed, everted and filled with Krebs-Ringer bicarbonate solution modified to contain either lactose or glucose (0.2 M) and 1×10^{-4} M calcium chloride. Each milliliter contained approximately 0.01 μc of Ca^{45}. The sacs were incubated in 3 ml of the same solution for 1.5 hours in the Dubnoff shaker at 37°C. The gas phase was 95% O_2—5% CO_2. For the studies lower in the gut, where active transport cannot be shown, the procedure was similar except that Ca^{45} was not added to the media used to fill the sacs and the sacs were incubated in 4 ml of solution. Sacs approximately 6-cm long were used. These were prepared from 3 different areas of the gut beginning approximately one-sixth, one-half and two-thirds of the length of the total intestine. In any study 2 adjacent sacs were compared, one with lactose and one with glucose in the medium, the order being varied from day to day. After incubation the contents of the sac were collected, the empty weight of the sac determined, and the Ca^{45} concentration of the contents determined. Active transport was recorded as the ratio of the concentration of Ca^{45} inside and outside of the sac. Rate of diffusion was calculated as the counts transferred per gram of tissue.

Differences between the active transport of animal pairs killed the same day were evaluated statistically by the *t* test. In evaluating the effects of lactose and glucose on passive transport, differences between adjacent sacs were used.

RESULTS AND DISCUSSION

As Schachter et al. (11) and Harrison and Harrison (13) have shown, transport of calcium against a concentration gradient can only be demonstrated in the most proximal portions of the gut of the rat. In the remainder of the intestine the process appears to have the characteristics of passive diffusion (13). Both processes are apparently stimulated by vitamin D.

The data upon active transport in the first 3 to 4 cm of the duodenum are shown in table 1. In very young animals, the transport was slightly, and probably significantly, stimulated by lactose as compared with glucose ($P < 0.05$). The ability to utilize lactose is rapidly lost so that animals 6 or more weeks old concentrate very little calcium in the lactose medium. It is known that the transport process requires energy; that the young animal has considerable lactase activity in the intestinal

[6] Thiamine·HCl, 200 mg; riboflavin, 400 mg; pyridoxine·HCl, 200 mg; Ca pantothenate, 1250 mg; niacinamide, 2000 mg; folic acid, 50 mg; biotin, 10 mg; vitamin B_{12}, 7.5 mg; and menadione, 5 mg mixed with 250 g of glucose.

TABLE 1

Effect of lactose on active intestinal transport of calcium [1]

Age	Body weight	No. of rats	Calcium transport (counts serosal media / counts mucosal media)			
			Low calcium diet		High calcium diet	
			Lactose	Glucose	Lactose	Glucose
weeks	*g*					
3–4	45–70	11	3.6 ± 0.15^a	3.0 ± 0.2^b	—	—
6–8	150–250	6	1.4 ± 0.13^c	3.7 ± 0.14^d	1.5 ± 0.15^e	2.7 ± 0.2^f

[1] Gut sacs prepared from the first 3 to 4 cm of the duodenum.
a > b $P < 0.05$.
c < d $P < 0.01$.
d > f $P < 0.02$.
c and e not significantly different.
[2] SE.

TABLE 2

Effect of lactose on passive intestinal transport of calcium [1]

Age	Body weight	Ca transport					
		Low calcium diet			High calcium diet		
		No. of animals	Lactose	Glucose	No. of animals	Lactose	Glucose
weeks	*g*	*count/min* × 10^{-3}/*g tissue*			*count/min* × 10^{-3}/*g tissue*		
3–4	45–70	8	16.2[a]	12.7[b]			
5–6	100–150	6	14.4[c]	12.3[d]	6	14.6	16.1
6–8	150–250	8	9.8	9.3	8	8.2	7.9

[1] Gut sacs prepared from the middle portion of the intestine.
a > b $P = 0.01$; SE of difference = 1.4.
c > d $P = 0.01$; SE of difference = 0.51.
Differences between other groups not statistically significant.

mucosa; and that this is rapidly lost as the animal ages (1, 2). It appears likely that the hydrolysis of lactose in the young animal would permit lactose to meet the energy requirements, but that this is not possible in older animals. However, the enhanced activity in the presence of lactose as compared with glucose would indicate an additional effect of lactose on the transport mechanism.

High calcium diets were not studied in the youngest animals since they were killed within the first week after receipt in the laboratory. The results obtained with older animals (table 2) confirm those of Dowdle et al. (16). The decreased transport in animals fed high calcium diets is apparently an adaptive mechanism reflecting decreased need. Nothing is known of the mechanism involved.

Passive transport in the lower sections of the gut apparently does not require energy (13). The effect of lactose compared with glucose in young animals appears quite clear-cut and was gradually lost with age. No effect of the amount of calcium in the diet upon this process was demonstrated. The passive transport of calcium does not appear to be uniform along the length of the intestine as shown in figure 1. Comparing segments from comparable animals at various levels in the gut, there was evidence of increased transport toward the distal end. In studies upon transport, such differences must be considered, and we have used alternate sections with glucose and lactose and with the order changed in different animals. These differences may also be of some significance in in vivo studies upon calcium absorption.

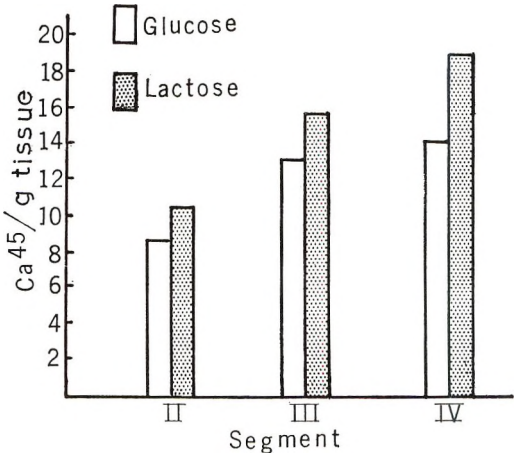

Fig. 1 Calcium transfer by the small intestine of young rats. Segment 1 (not shown) represents the proximal portion of the intestine where calcium is transported against a concentration gradient. Segments II, III, and IV were taken at approximately one-third, one-half and two-thirds the length of the intestine. Data reported are counts per minute $\times 10^{-3}$ of Ca^{45} appearing in the everted gut sac/g of intestine.

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Effects of Dietary Creatine and Related Compounds on Tissue Glycogen Deposition in Rats[1,2]

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ABSTRACT Rats fed a purified diet containing added glycine maintain liver glycogen at a higher level following cold water swimming stress than rats fed the diet without glycine. Following recovery from the stress the glycine-fed animals deposit, and the control-fed rats lose liver glycogen. Ingestion of either diet containing one per cent added creatine abolishes this "glycine effect" and liver glycogen levels before the stress are less than one-half those observed in animals prefed the diets without supplemental creatine. The ingestion of N-methyl glycine, N,N-dimethyl glycine or guanido acetic acid in the diets rather than creatine leads to similar results. However, creatinine ingestion inhibits the "glycine effect" but does not lower the pre-stress liver glycogen level. It is assumed that creatine and some related compounds inhibit the increased gluconeogenesis brought about by glycine feeding followed by stress, and also inhibit some step in the deposition of liver glycogen in the absence of stress.

Creatine, as a dietary supplement, has been studied in several species of animals and with respect to various biological actions. Baby pigs fed a diet supplemented with creatine showed no improvement in growth rate and no increase in either muscle size or creatine content (1). Parenteral administration of creatine to suckling pigs resulted in neither growth improvement nor increased muscle creatine levels (2). A linear increase in muscle creatine and a slight acceleration of growth was observed in chicks fed a diet supplemented with creatine (3). In rats (4, 5) and in chicks (6), dietary creatine depressed kidney transamidinase activity. The activity of this enzyme in the kidney was suggested as a controlling mechanism for creatine biosynthesis (4, 7). Other workers (8) observed no effect of previous creatine feeding on the ability of the isolated perfused rat liver to synthesize creatine. In mice, kidney transamidinase activity was depressed after ingesting a creatine-containing diet and the depression was removed after the animals were fed the diet to which glycine had been added (9).

In another connection it has been observed in our laboratory that creatine ingestion by rats resulted in a marked effect on carbohydrate metabolism under the specific experimental conditions used.

Previous work demonstrated that rats pre-fed a diet containing added glycine maintained carbohydrate reserves at a higher level than rats fed the diet without added glycine following the stress of a 24-hour fast (10) or the stress of a large dose of insulin (11). Results of analysis of major tissues for free and combined glycine established that insufficient free glycine was present for the extra glycogen of glycine fed rats to have been formed through direct conversion of the amino acid (12). During fasting in rats pre-fed the glycine diet, the net negative nitrogen balance was of such a magnitude as to constitute corroboration for earlier postulates (10, 12) that glycine feeding followed by stress resulted in increased gluconeogenesis, probably through an effect on the adrenal cortex, since the effect could not be demonstrated in adrenalectomized rats (10).

Following the stress of cold water swimming, rats pre-fed the glycine diet showed higher liver glycogen levels than the control-fed animals. During recovery following the stress the glycine-fed rats demonstrated a marked redeposition of liver

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[2] A preliminary report was presented at the Pacific Slope Biochemical Conference in Seattle, September, 1962.

glycogen, whereas those pre-fed the control diet showed no increase (13). This extra carbohydrate synthesis following stress in glycine-fed animals, has been termed the "glycine effect." Adrenalectomized rats did not demonstrate the "glycine effect" after cold water swimming stress unless replacement therapy (cortisol) was instituted (14).

The present report deals with experiments showing that the ingestion of creatine under the conditions used abolished the "glycine effect." Furthermore, the initial levels of liver glycogen (previous to swim stress) were less than one-half those of animals pre-fed the same diet without creatine. The ingestion of the diet supplemented by any one of several compounds related to creatine and thus to glycine afforded somewhat similar results.

EXPERIMENTAL PROCEDURE

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

Diets and feeding. Two basic diets with the following compositions were used. Control ration: (in per cent) casein, 16; salt mixture,[3] 5; brewer's yeast,[4] 10; cod liver oil, 2; cottonseed oil, 5; white corn dextrin, 54; and glucose, 8. Glycine ration: 10% of the dextrin of the control diet was replaced by an equal weight of glycine. These 2 diets were used in this form and after supplementation with 1% creatine, creatinine, N-methyl glycine, N,N-dimethyl glycine or guanidoacetic acid.

At the start of an experiment, rats were removed from the laboratory chow[5] and fed the control diet for 24 hours to accustom them to purified rations. Some of the animals were maintained with this diet, and others were changed to one of the other diets for an additional 24 hours. At this time the final meal was given as a slurry by stomach tube as described previously (13) in order to equalize food intake and to control the time of food intake prior to the 4-hour period before the rats were stressed. Each experiment involved one or more animals pre-fed the control ration, one or more pre-fed the glycine ration, and one or more pre-fed one of the supplemented diets. All of the rations were eaten satisfactorily and total intake was maintained at 18 to 19 g for the final 24-hour feeding period including that given by gavage. The intake during 24 hours of any one of the supplements used at the 1% level amounted to about 185 mg.

Stressing. Four hours after the stomach-tubed meal animals were either killed to obtain tissues for analysis or were stressed in cold water. The rats were made to swim individually for 10 minutes in a large sink containing water about 30-cm deep which was maintained at 14°C. After 30 minutes rest in individual cages at 25°C, they were made to swim another 10 minutes and rested again for 30 minutes. Following a final 10-minute swim they were killed at once to obtain tissues or were allowed to recover at 25°C in individual cages for three hours before they were killed. Since the animals were cold and shrivering during the 30-minute rest periods, the stress actually totaled 90 minutes.

Tissue preparation. Following intraperitoneal anesthesia with pentobarbital sodium[6] (8 mg/200 g body weight), the liver was removed, blotted and minced by one pass through a custom built "garlic press." Aliquots were added to tared tubes containing 30% KOH. This procedure was accomplished in less than 60 seconds after opening the abdomen. The whole right gastrocnemius muscle was immediately removed and minced with scissors into similar tubes.

Methods. Glycogen was determined by the method of Good et al. (15) except that the isolated glycogen was redissolved in water and reprecipitated with alcohol. Glucose was estimated by the Somogyi modification of the Shaffer-Hartmann method (16) after hydrolysis of the glycogen for 2.5 hours in NH_2SO_4 at 100°C. Blood sugar was estimated by the Somogyi technique (17) on heart blood drawn with a syringe containing heparin.

RESULTS AND DISCUSSION

Initial levels of liver glycogen, 4 hours after stomach tubing and without stress, were the same in animals pre-fed the glycine ration or the control ration (table 1). During the swimming stress the animals

[3] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland.
[4] Squibb Flavored Yeast, E. R. Squibb and Sons, New York.
[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[6] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.

TABLE 1

Liver and muscle glycogen in rats without stress, after swimming stress and following recovery from stress (animals pre-fed the control diet, the glycine diet or this diet supplemented with creatine or related compounds) [1]

Diet pre-fed	Glycogen					
	No stress		After swim stress		Following recovery from stress	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
	% wet weight		*% wet weight*		*% wet weight*	
Control	3.73 ± 0.15 [2]	0.55 ± 0.02	0.97 ± 0.05	0.11 ± 0.01	0.86 ± 0.09	0.25 ± 0.02
Glycine	3.49 ± 0.12	0.56 ± 0.02	1.70 ± 0.09	0.16 ± 0.02	2.95 ± 0.09	0.27 ± 0.02
Glycine + 1% creatine	1.42 ± 0.12	0.40 ± 0.01	1.29 ± 0.13	0.10 ± 0.01	0.39 ± 0.14	0.25 ± 0.01
Glycine + 1% N-methyl glycine	2.91 ± 0.12	0.53 ± 0.04	1.56 ± 0.11	0.14 ± 0.02	0.99 ± 0.19	0.27 ± 0.02
Glycine + 1% N,N-dimethyl glycine	1.93 ± 0.15	0.53 ± 0.04	1.28 ± 0.05	0.05 ± 0.01	0.39 ± 0.02	0.15 ± 0.02
Glycine + 1% guanido acetic acid	2.51 ± 0.17	0.50 ± 0.01	1.53 ± 0.11	0.08 ± 0.02	0.64 ± 0.17	0.25 ± 0.05
Glycine + 1% creatinine	3.43 ± 0.09	0.52 ± 0.05	1.76 ± 0.09	0.12 ± 0.02	0.34 ± 0.06	0.18 ± 0.03

[1] Each figure is an average value from 5 or more rats.
[2] SE of mean.

fed glycine maintained liver glycogen at a higher level than did the controls. During the recovery period following the stress, the glycine-fed rats resynthesized and deposited liver glycogen, whereas the control fed rats did not. This extra carbohydrate formation constitutes the "glycine effect." Increased gluconeogenesis has been postulated as the mechanism accounting for this effect (12, 13).

The marked action of creatine ingestion (creatine-glycine diet) on liver glycogen levels is also illustrated by the data in table 1. The initial liver glycogen level in these rats was less than one-half that observed in rats pre-fed either the control or the glycine ration. There was no loss during the swim stress but there was a severe depletion in the 3-hour recovery period after stress. Thus, the "glycine effect" was absent in these animals.

The rats pre-fed the creatine-glycine diet were the only animals studied that did not show a loss of liver glycogen during the swimming stress. Muscle glycogen was also low initially in these animals and the loss during stress was similar to that noted in other groups of animals (table 1). The energy requirement in these animals during stress must have been met to a greater degree by other sources than liver and muscle glycogen.

The elevated blood sugar (table 2) following the swimming stress in the animals pre-fed the glycine diet may reflect the gluconeogenesis preceding the deposition of liver glycogen. The control-fed animals and the creatine-glycine fed rats did not show this elevation of blood sugar. Urine removed from the bladder of creatine-glycine fed rats at sacrifice showed only traces of sugar with Benedict's qualitative urine sugar reagent.

Of all the animals studied those pre-fed the creatine-containing diets performed the poorest in the swim stress. It was frequently necessary to place a hand under these animals and give them a momentary

TABLE 2

Blood sugar in rats without stress, after swimming stress and following recovery from stress (animals pre-fed the control diet, the glycine diet or the creatine-glycine diet) [1]

Diet pre-fed	Blood sugar		
	No stress	After swim stress	Following recovery from stress
	mg/100 ml		
Control	132±2.4 [2]	124±5.5	115±0.9
Glycine	127±7.5	181±3.5	128±0.8
Creatine	120±8.1	147±9.6	97±2.3

[1] Each figure is an average value from 5 or more rats.
[2] SE of mean.

lift, especially during the final 10-minute swim period.

Creatine, under the conditions fed, showed no other adverse effects on the rats as judged by short time food consumption and growth. Averages for 3 to 5 animals for 7 days' growth and one day's food consumption of different diets were as follows: (in grams) control diet, 32 and 18.7; creatine-control diet, 31 and 18.7; glycine diet, 29 and 18.3; creatine-glycine diet, 28 and 18.5. Growth during the 24 hours with the different experimental diets averaged out to almost identical figures. The low initial levels of liver and muscle glycogen as a result of creatine ingestion bear no apparent relation to other metabolic changes reported by others in animals fed extra dietary creatine.

To determine whether this effect on carbohydrate metabolism is unique to the creatine molecule, several compounds related to creatine, and thus to glycine, were studied as dietary supplements under the same experimental conditions.

Animals pre-fed the glycine diet supplemented with 1% N-methyl glycine, N,N-dimethyl glycine or guanidoacetic acid had lower initial liver glycogen values than animals pre-fed the unsupplemented glycine ration (table 1). During the stress there was loss of liver glycogen, and a further decrease occurred during the recovery period in each instance. Thus, the ingestion of any one of these compounds, like the ingestion of creatine, abolished the "glycine effect." Muscle glycogen values were not materially different from the values observed in animals pre-fed the glycine diet at any of the times studied as a result of ingesting these diets. The increase in muscle glycogen during the recovery phase, at least in part, must reflect conversion of pyruvic acid to glucose following the severe stress.

Creatinine ingestion did not lower the initial level of liver glycogen, and the decrease as a result of stress was the same as noted in animals fed the glycine diet without added creatinine. However, the redeposition of liver glycogen during the recovery period was blocked as was found when the other supplements were used. This raises the possibility that some difference exists in the mechanisms involved in the altered carbohydrate metabolism from creatine ingestion for instance, with and without stress.

The observed effect of ingested creatine and related compounds on initial levels of liver glycogen is not dependent upon the simultaneous ingestion of glycine. The experiments were all repeated using the control ration supplemented with each of the compounds studied. In every case initial glycogen levels showed a similar pattern to those observed when the supplemented glycine diet was used. Again, there was no decrease in the initial level of liver glycogen in animals pre-fed the creatinine-containing ration. During the stress the decrease in liver glycogen was about the same or more marked with these supplemented rations than in animals pre-fed the supplemental glycine diet. One exception was found; with the creatinine diet the decrease was much less marked. Following recovery the liver glycogen levels were similar or lower than the corresponding value from animals fed the supplemental glycine diet. Muscle glycogen values were similar at all 3 times studied to those noted in rats pre-fed the supplemented glycine ration.

Considerable data point to increased glyconeogenesis as the mechanism accounting for the "glycine effect." The abolition of this effect due to ingestion of creatine and related compounds may reflect inhibition of some step in the formation of glucose or in the deposition of this glucose as liver glycogen. On this basis it is possible that the low levels of liver glycogen initially may also be related to inhibition of a part of the process of deposition. Other explanations to account for

these results such as lack of availability of substrate and membrane permeability alterations, or increased glycogenolysis are not ruled out, however, by the data available.

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A Mechanism of Interaction between Dietary Protein Levels and Coccidiosis in Chicks[1]

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ABSTRACT Chicks were used to study a mechanism of interaction between dietary protein levels and coccidiosis. The results of the study indicate mortality from coccidiosis is less among groups of chicks fed a protein-free diet for 48 hours, or a 5% protein diet for 14 days before inoculation with coccidia, than among chicks fed a 20% protein diet. Starvation had the same effect as the protein-free diet. Observation of gross cecal lesions also indicates less infection in chicks fed a protein-free diet than in chicks fed a 20% protein diet. Low protein diets also reduced intestinal trypsin activity. A positive correlation was shown between dietary protein level and intestinal trypsin activity and between intestinal trypsin activity and degree of infection of chicks with coccidiosis. Addition of trypsin to the inoculum of the chicks fed a protein-free diet resulted in an infection as severe as when the chicks were fed a 20% protein diet. Since trypsin has been found to be essential for the establishment of coccidiosis infection, it is proposed that the effect of low protein diets in decreasing the severity of coccidiosis is due to the effect of these diets on intestinal trypsin activity.

The effect of dietary protein levels on resistance or susceptibility to bacterial infection has been found to be unpredictable. In some instances increasing protein levels increased resistance (1); in others, increased susceptibility (2); and in at least one instance (3) was found to have both effects or neither depending upon the presence of other dietary factors.

Coccidiosis has been studied in relation to dietary protein levels by Allen (4) and Jones (5) who reported no marked effect on coccidiosis of chickens. In preliminary experiments in this laboratory, it was observed that chicks fed a protein-free diet for 2 weeks were much less susceptible to coccidiosis than were chicks fed a diet containing 20% protein.[2] The purpose of the work presented in this report was to seek an explanation of this observation.

EXPERIMENTAL

The chicks used in these studies were White Plymouth Rocks obtained from a commercial hatchery. They were housed in a conventional battery brooder and fed a commercial diet until they were given the experimental diets, at which time they were placed in broiler batteries with raised wire floors. The composition of the experimental diets is presented in table 1. The protein content of the various diets was adjusted by changing the soybean meal level. The methionine level was changed in proportion to the protein level. A constant energy level was maintained by adjustment with fat and fiber.

Uninfected chicks were maintained on the same regimen as the infected. Since there was no mortality in the uninfected animals, all mortality must be ascribed to the infection.

Trypsin activity in the intestinal tract was determined by a modified spectrophotometric method described by Kunitz (6). A section of the lower small intestine just above the cecum was taken from each chick. The section was from 5 to 10 cm long so as to make the sections approximately the same size from each chick. The section was slit open and swirled in 100 ml of 0.0025 M HCl. This solution was filtered through filter paper and 2 ml of the filtrate was incubated with 2 ml of a 1% solution of casein in Sorensen phosphate buffer at pH 7.6 in a water bath at 37°C for 20 minutes. Six milliliters of 5% trichloroacetic acid were mixed with each sample at this time and allowed to stand for one hour. The sample was filtered and the

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[1] Published with the approval of the Director of the Experiment Station as Paper no. 1705 of the Journal Series.

[2] Unpublished data, F. R. Craig and W. L. Payne.

TABLE 1

Composition of basal diets

	Protein				
	0	5	10	20	30
	%	%	%	%	%
Soybean meal (50% protein)	0	10	20	40	60
Glucose [1]	83.2	77.7	72.3	53.3	29.6
Defluorinated rock phosphate	3.2	3.2	3.2	3.2	3.2
NaCl	0.5	0.5	0.5	0.5	0.5
Vegetable oil [2]	1.0	1.0	1.0	3.0	6.0
Fiber [3]	11.9	7.4	2.7	0	0
	mg/kg	*mg/kg*	*mg/kg*	*mg/kg*	*mg/kg*
$MnSO_4$	220	220	220	220	220
Vitamin mix [4]	1980	1980	1980	1980	1980
DL-Methionine	0	75	150	300	450

[1] Cerelose, Corn Products Refining Company, New York.
[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Supplies per kilogram of diet: (in milligrams) thiamine, 3.5; riboflavin, 5.7; folic acid, 1.1; α-tocopheryl acetate, 22.0; menadione sodium bisulfite, 0.79; biotin, 0.18; pantothenic acid, 18.3; niacin, 52.3; pyridoxine, 5.7; and cyanocobalamin, 8.8 μg; vitamin A, 11,000 USP units; and vitamin D, 1,980 ICU.

optical density at 280 mμ was determined on a Cary recording spectrophotometer.

A standard curve was obtained by using trypsin (2 × crystallized) 50% $MgSO_4$[3] as a standard with the activity of 1 μg being defined as a trypsin unit. The protein content of the filtrate of the intestinal contents was determined by measuring the optical density at 260 and 280 mμ and the trypsin activity was expressed in trypsin units per milligram of protein.

The results of the experiments were evaluated for statistical significance by analysis of variance. Arcsin /percentage transformation was used on all of the percentage data analyzed (7).

RESULTS AND DISCUSSION

The first experiment was conducted to test the effect of dietary protein levels on the resistance of chickens to coccidiosis. The protein levels varied from zero to 30% in 5% intervals. These diets were fed to 4-week-old chicks for 2 weeks before and one week after oral inoculation with 200,000 sporulated oocysts of *Eimeria tenella*.[4] One week after inoculation the chicks were fed a 20% protein commercial ration. Mortality and gross cecal lesions were observed. Mortality occurred between the fifth and ninth days post-inoculation. The results of this study are presented in table 2. Statistical analysis of the mortality and lesion scores showed a significant decrease in both at the zero and 5% protein levels. Furthermore, the chicks fed the higher levels of protein (15 to 30%) developed symptoms of coccidiosis, bloody excreta and lethargy, earlier after inoculation.

TABLE 2

Mortality and gross cecal lesion scores of chicks fed different levels of protein and infected with Eimeria tenella

Protein in diet [1]	Mortality [2]	Gross cecal lesion scores [3]
%	%	
0	0	1.5
5	0	1.7
10	10	3.0
15	15	2.7
20	15	3.3
25	5	3.1
30	20	3.3

[1] Diets fed for 2 weeks before and 1 week after inoculation.
[2] Twenty birds were fed each diet.
[3] Mean of 10 birds scored zero for no lesions to 4 for most severe lesions.

In seeking an explanation of these results, attention was directed to the life cycle of the coccidia. It has been found that excystation of the sporozoites from the oocyst in dependent upon the presence of trypsin (10). Levine (8) and Ikeda (9) observed that chicks with ligated pancreatic ducts did not become infected when inoculated orally with sporulated oocysts. When trypsin was added to the inoculum, infection occurred (10).

[3] Nutritional Biochemicals Corporation, Cleveland.
[4] Donated by Merck and Company, Inc., Rahway, New Jersey.

Trypsin activity of the pancreas has been found to be reduced by low levels of dietary protein (11, 12). It was possible, therefore, that the effect of dietary protein levels on coccidiosis was a reflection of the effect of these levels on intestinal trypsin activity. To assess the effect of level of protein on trypsin activity, an experiment was conducted in which intestinal trypsin activities of chicks fed diets containing 5, 20, and 30% soybean protein and a 20% protein commercial diet were determined. The results (table 3), show a significant reduction in the trypsin activity of the 5% protein fed group when compared with those fed higher protein levels. These data supported the possibility that the effect of the low protein diet on trypsin activity was causally related to the effect on mortality from coccidiosis.

The effect of low dietary protein levels on intestinal trypsin activity was most probably a rapidly occurring phenomenon. To determine how rapid, one group of chicks receiving the commercial diet was changed to a protein-free diet and another group was starved. After 24, 48, and 72 hours, 4 chicks from each treatment, along with four continuing to receive the commercial diet, were killed and the intestinal trypsin activity determined. The results are presented in table 4. Within 24 hours the intestinal trypsin activity of those chicks receiving the protein-free diet or those chicks starved was much lower than that of the 20% protein commercial ration.

These observations indicate that if intestinal trypsin activity and susceptibility to coccidiosis were causally related a short period of feeding a protein-free diet or starvation should suffice to produce the effect. Accordingly, an experiment was conducted in which the chicks were either

TABLE 3

Trypsin activity of intestinal section of chicks fed various levels of protein in the diet

Time fed diets	Diets			
	% Protein in diet			
	5	20	30	20 (commercial)
days	*trypsin units/mg protein* [1]			
14	184	472	475	433
16	116	644	456	395

[1] Each value is a mean of 5 chicks tested.

TABLE 4

Trypsin activity of intestinal section of chicks starved and of chicks fed a protein-free and a 20% protein commercial ration

Time fed diet	Diet		
	Starved	Protein-free	20% Protein (commercial)
hours	*trypsin units/mg protein* [1]		
24	7	4	328
48	15	18	813
72	18	14	682

[1] Each value is a mean of 4 chicks tested.

TABLE 5

Mortality of chicks from coccidiosis when starved, fed a protein-free diet, or a 20% protein diet

Diet	Days post-inoculation				
	5	6	7	8	9
	Cumulative % mortality [1]				
Starved [2]	3	10	27	27	27
Protein-free [3]	0	17	29	29	29
20% Protein [3]	32	45	50	55	55

[1] Mean value of 2 groups of 12 chicks each.
[2] Starved for 48 hours before and 96 hours after inoculation.
[3] Diets fed for 48 hours before and one week after inoculation.

fed the protein-free diet or starved for 48 hours prior to inoculation with sporulated oocysts of *Eimeria tenella*, *Eimeria maxima*, *Eimeria brunetti*, and *Eimeria acervulina*.[5] Ninety-six hours after inoculation the starved groups were fed the commercial diet. Seven days after inoculation the groups fed a protein-free diet were fed the commercial diet. The results of that study are presented in table 5. A significant reduction in mortality was noted in the starved chicks and those fed the protein-free diets even though this treatment was started only 48 hours before inoculation.

A similar experiment in which the effects of the protein-free diet fed for 48 hours before inoculation and the 20% protein soybean meal basal diet were compared yielded similar results (table 6). In this experiment the inoculum contained, in addition to those mentioned above, sporulated oocysts of *Eimeria hagani*, *Eimeria necatrix*, *Eimeria praecox*, and *Eimeria mivati*.[6]

The results of these 2 studies coupled with those of a previous experiment (table

[5] See footnote 4.
[6] Donated by Dom and Mitchell, Inc., Opelika, Alabama.

TABLE 6

Mortality of chicks from coccidiosis when fed a protein-free or a 20% protein diet

Diet	Days post-inoculation				
	5	6	7	8	9
	Cumulative % mortality [1]				
Protein-free [2]	0	22	55	65	75
20% Protein [2]	0	75	90	95	95

[1] Each value a mean of 4 groups of 10 birds each.
[2] Diets fed for 48 hours before and one week after inoculation.

4) strengthened the theory that the dietary protein effect on susceptibility to coccidiosis was mediated by way of the intestinal trypsin activity. Therefore, a more direct test of the influence of protein levels and trypsin activity on susceptibility to this infection was conducted. In this test chicks were fed either a protein-free or a 20% protein diet for 48 hours before inoculation with the sporulated oocysts of the 8 species of *Eimeria* shown above. The special inoculum was prepared both with and without 1% added trypsin[7] and given to 2 groups of 10 chicks/diet. The trypsin was prepared as a 2% solution in Sorensen's phosphate buffer at pH 7.6 and mixed with equal volumes of the normal inoculum. In the control, Sorensen's phosphate buffer was mixed with equal volumes of the normal inoculum, but trypsin was not added.

The results of this study are presented in table 7. The mortality was significantly reduced in the chicks fed the protein-free diet, and the addition of trypsin to the inoculum increased the mortality to that of the 20% protein-fed chicks. The addition of trypsin to the inoculum of the 20% protein-fed group did not result in a significant increase in mortality.

The results of this study establish more clearly the relationship between dietary protein levels, trypsin activity, and susceptibility to coccidiosis. In this infectious model, the effect of low dietary protein levels was a definite reduction of the normal trypsin activity of the intestinal tract which is necessary for normal excystation of the oocysts. Without excystation infection does not occur. It should be noted that even the chicks fed the low protein diets still had some trypsin activity in the intestinal tract which may account for the infection that was observed in these groups.

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[7] Trypsin 1:300, Nutritional Biochemicals Corporation, Cleveland.

TABLE 7

Effect of protein-free and 20% protein diets on mortality from coccidiosis with and without added trypsin in the inoculum

Diet [1]	Inoculum	Days post-inoculation			
		5	6	7	8
		Cumulative % mortality [2]			
Protein-free	control	0	10	40	45
Protein-free	trypsin added	5	60	70	85
20% Protein	control	55	80	85	85
20% Protein	trypsin added	75	95	95	95

[1] Diets fed for 48 hours before and one week after inoculation.
[2] Mean values of 2 groups of 10 chicks each.

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Effects of Egg Oil, Cholesterol, Cholic Acid and Choline upon Plasma and Liver Lipids[1]

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ABSTRACT Young adult female rats of the Wistar strain were fed diets containing 27% egg oil or a mixture of butter fat and olive oil with the same iodine number as the egg oil but with different fatty acid content. The other variables were 0.1 or 0.3% choline chloride, 1.0% cholic acid, and 1.29% cholesterol — the level of cholesterol present in the egg oil. The experiments were of 5 to 10 weeks' duration. Rats fed the rations containing the mixture of fats with cholesterol and either level of choline had plasma and liver cholesterol and liver lipid levels that were significantly augmented over those receiving the egg oil. Cholic acid affected an increase in the cholesterol and lipid values of the rats receiving both the fat mixture with cholesterol and the egg oil. The liver lipids and cholesterol decreased when the higher level of choline was fed in conjunction with the mixture of fats with cholesterol and cholic acid. The liver lipids and cholesterol were threefold and twelve-fold, respectively, greater for the rats receiving egg oil than for those fed the fat mixture without cholesterol or cholic acid. Groups given the fat mixture with cholesterol and cholic acid had far higher values than did any of the other groups.

A number of investigators have suggested that certain natural fats contain substance(s) other than cholesterol that increase serum cholesterol. Messinger and co-workers (1) reported that the elevation of serum cholesterol in human subjects fed diets containing egg yolk powder or cream was greater than could be accounted for by the cholesterol contained therein. They concluded, "there is a substance as yet unidentified in egg yolk which elevates the cholesterol level of human serums." Mayer and co-workers (2) failed to note demonstrable change in the plasma cholesterol level when 800 mg of cholesterol/day were given in the form of egg yolk. This observation is in agreement with the results of Keys et al. (3) and Hildreth and co-workers (4). Mayer and co-workers (2) have pointed out that the lack of agreement between the work carried out in their laboratory and that of Messinger et al. (1) could be accounted for by the higher level of egg yolk powder, and the cholesterol contained therein, fed by Messinger et al. The saturated fatty acids in egg yolk could also contribute to the hypercholesterolemia. The cholesterol consumed by Messinger's subjects was at least 4 times that of Mayer's. Bronte-Stewart et al. (5) confirmed the observations of Messinger, giving 10 eggs/day — an amount which supplied essentially the same quantity of cholesterol as received by Messinger's subjects. Portman (6), in a review on factors influencing serum cholesterol levels states: "It appears that the form in which dietary cholesterol occurs is of importance; for example, egg yolk appears to have greater hypercholesteremic activity than does an equivalent amount of crystalline cholesterol. This latter observation may be related to the nonsterol portion of the egg yolk, however. The nature of the basic ration in which the sterol is included may also be of importance."

Wells and Bronte-Stewart (7), working with a single human subject, have recently reported that the cholesterogenic entity in egg yolk resided in the acetone-soluble fraction. Neither the unsaponifiable nor the saponifiable fractions gave an increase in serum cholesterol when fed separately. The 2 fractions were active when fed together but a delay of 6 hours in feeding one or the other nullified this effect. A fat of like iodine number with added cholesterol gave similar effects.

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Rowsell et al. (8) fed diets to swine in which 33% of the calories was replaced by egg yolk or by butter fat. Controls received the non-supplemented basal diet. The swine fed egg yolk had approximately 6 times as much aortic atherosclerosis as did the controls, and the butter-fed swine 3 times as much. The group fed egg yolk showed a significant increase in serum cholesterol and phospholipid levels. The swine fed butter fat showed only a slight increase in serum lipid levels.

Hegsted et al. (9) studied the effects of the interaction of cholesterol, cholic acid, and the type of fat on serum cholesterol and vascular sudanophilia in the rat, using 3 levels of cholic acid and 3 levels of cholesterol in all of their combinations. They found that cholic acid had a relatively greater effect than did cholesterol in producing an increase in serum cholesterol. Dose-level responses appeared to indicate an interaction of cholesterol and cholic acid.

The effect of choline on serum cholesterol was studied by Mayfield and Roehm (10) who observed that female rats receiving no choline for 18 days had serum cholesterol values that were essentially the same as those of rats fed varying levels of choline ranging from 30 to 350 mg/100 g diet. Male rats maintained with a choline-free diet had significantly lower serum cholesterol than those receiving the rations supplemented with choline. However, the male and female rats receiving the lower levels of choline — zero, 30 or 60 mg/100 g diet — had lower serum cholesterol when compared with those receiving the higher levels of choline — 100, 250 or 350 mg/100 g diet.

Olson et al. (11) observed hypocholesterolemia, hypolipemia and hypobetalipoproteinemia in rats fed diets deficient in choline and choline-precursors. These effects were prevented by 0.3% choline in the diet and partially prevented by rations containing 18% casein. The level of dietary fat ranging from 6 to 42% and including butter fat, corn oil and lard did not modify the effects of choline upon serum lipids.

The present study deals with the effects of egg oil and of a fat mixture upon plasma cholesterol and liver total lipids and cholesterol of young adult female rats.

MATERIALS AND METHODS

Diets. The purified diets contained 18% casein, 4% salts (Hegsted (12)), optimal vitamins,[2] dextrose and 27% fat (table 1). The level of fat corresponded to the fat content of the average American diet (13). The rations contained either egg oil or a mixture of fats of like iodine number and with or without added cholesterol or cholic acid or both. Choline chloride was fed at 2 levels.

The egg oil was extracted from baker's dried whole egg powder with diethyl ether for 16 hours in a Soxhlet extractor. The residual ether was distilled off; the brown viscous oil that remained was free of peroxides. It was stored at 4°C. The egg oil was analyzed by gas-liquid chromatography for individual fatty acids using a column packing of diethylene glycol succinate on chromosorb W in the Barber-Colman gas chromatograph. The fatty acid compostion of the egg oil is shown in table 1. The iodine number as determined by the method of Hanus (14) was 72.

The fat mixture was composed of a 21:6 ratio of olive oil-butter fat. The butter fat was prepared from unsalted butter by heating until molten and centrifuged at 800 rpm for 15 minutes to remove the water and milk solids. The butter fat was frozen until used. A single batch of olive oil stored at 4°C was used. The iodine number for the butter fat was 32 and the olive oil was 85; the resulting mixture of olive oil and butter fat had an iodine number of 73. Although the iodine number indicated a like degree of unsaturation when compared with the egg oil, the content and proportion of individual fatty acids were not the same. The primary difference was in the presence of short-chain fatty acids in the olive oil-butter fat mixture as contrasted with their absence from the egg oil. A higher content of 16:0, 16:1, and 18:2 fatty acids was present in the egg oil.

The fat mixture was fed with and without cholesterol. The fat mixture with added cholesterol contained a total of

[2] Vitamins through the courtesy of Merck, Sharp and Dohme Research Laboratories, Inc., Rahway, New Jersey, and Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Diet composition

	g/100 g		Iodine no.
Casein	24	Egg oil	72
Fat	27	Olive oil	85
Salts [1]	4	Butter fat	32
Dextrose to	100	Fat mixture	73

Fatty acid composition			Vitamin addendum	
Fatty acids	Egg oil	Fat mixture		
	%	%		mg/100 g
4:0 [2]	—	0.32	Thiamine	1
6:0	—	0.03	Riboflavin	2
8:0	—	0.51	Pyridoxine·HCl	1
10:0	—	0.89	Ca pantothenate	10
10:1	—	0.16	Niacinamide	10
12:0	—	1.31	Inositol	5
14:0	0.03	3.39	*p*-Aminobenzoic acid	30
14:1	—	0.48	Biotin	0.05
16:0	29.50	14.34	Folic acid	0.2
16:1	3.18	1.60	α-Tocopherol	14.2
18:0	9.62	6.12	Vitamin B_{12}	0.01
18:1	47.90	62.88	Menadione	14.2
18:2	9.55	6.61		IU/100 g
19:0	—	1.36	Vitamin A	150
Polyunsaturated	9.55	6.61	Vitamin D	15
Monounsaturated	51.08	65.12		
Saturated	39.42	28.02		

[1] Hegsted et al. (12).
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.

1.29% cholesterol which corresponded to the cholesterol content of the egg oil as determined by the Sperry-Webb method (15). This quantity was provided by the butter fat containing 0.02% cholesterol and by 1.27% of added cholesterol.

As bile acids are known to increase the absorption of cholesterol, 1% cholic acid was added to some of the diets. Choline chloride was fed as 0.1 or 0.3% of the rations.

The dietary groups were as follows: 1) egg oil with 0.1% choline; 2) egg oil with 0.3% choline; 3) fat mixture with 0.1% choline; 4) fat mixture with 0.3% choline; 5) fat mixture, cholesterol and cholic acid with 0.1% choline; 6) fat mixture, cholesterol and cholic acid with 0.3% choline; 7) fat mixture and cholesterol with 0.1% choline; 8) fat mixture and cholesterol with 0.3% choline; 9) fat mixture and cholic acid with 0.1% choline; 10) fat mixture and cholic acid with 0.3% choline; 11) egg oil and cholic acid with 0.3% choline; and 12) egg oil with 0.3% choline.

This study was carried out in 3 experiments. Experiment 1 (groups 1–6) compared the effects of egg oil with the mixture of fat with and without added cholesterol and cholic acid. Experiment 2 (groups 7–10) compared the individual effects of cholesterol and cholic acid fed with the fat mixture. Experiment 3 (groups 11 and 12) studied the effects of cholic acid upon the absorption of cholesterol as contained in egg oil.

Animals. Young adult female rats of the Wistar strain were used. Groups of 10 rats each were used in experiment 1. The animals averaged 214 g in weight. They were maintained with their respective diets for 10 weeks. Groups of 3 rats each were used in experiment 2. The rats averaged 240 g in weight and were fed the diets for 8 weeks. Groups of 5 rats each were used in experiment 3. They averaged 214 g in weight and the duration of the experiment was 5 weeks.

The rats in all groups were housed in individual cages in an air conditioned room kept at 21.8°C. The diets were fed

ad libitum and food consumption was recorded 3 times weekly. The rats were weighed semi-weekly.

The animals were killed after the period stated by an intraperitoneal injection of pentobarbital sodium.[3] The blood was taken from the deeply anesthetized animals by heart puncture and the livers were removed immediately. The livers were wiped with tissue and weighed. A piece of liver, approximately 2 cm^3 was placed in 30% formalin for histological studies. The remainder was wrapped in Saran Wrap,[4] frozen and stored until used for biochemical determinations.

Methods. Plasma cholesterol was determined by the method of Sperry-Webb (15). The frozen livers were brought to room temperature and ground in a mortar with sodium sulfate. The total lipids were extracted from an aliquot of the wet liver with petroleum ether. An aliquot of the petroleum ether fraction was used for the cholesterol determination (15).

RESULTS AND DISCUSSION

Experiment 1. The food consumption averaged the same for all groups, 12 g daily. The rats receiving the egg oil (groups 1 and 2) made somewhat greater weight gains than those fed the fat mixtures with or without any variants (groups 3–6). The animals made gains expected for females of their age and did not vary appreciably from group to group. All data for experiment 1 are shown in table 2.

The majority of the rats receiving cholesterol and cholic acid with either level of choline (groups 5 and 6) had bloody noses and tails. Several of the animals in these groups excreted urine of gelatinous consistency. The visceral fat in some of these rats was not white and translucent, as in normal animals, but rather brown and opaque in appearance. Despite rough fur and the abnormalities mentioned, the rats were otherwise normal in appearance and activity.

The plasma cholesterol of the rats fed the egg oil diets (groups 1 and 2) was not affected by tripling the choline in their rations. The cholesterol was higher for the animals receiving the egg oil ($P < 0.01$) than for those fed the fat mixture without added cholesterol and cholic acid (groups 3 and 4). The increased choline produced a significant lowering of the cholesterol in the latter groups ($P < 0.05$). The rats receiving the fat mixture with cholic acid and cholesterol (groups 5 and 6) had extremely high levels of plasma cholesterol: namely, a fivefold increase over groups 1 to 4, inclusive ($P < 0.01$). Increasing the choline from 0.1% to 0.3% was without effect upon the plasma cholesterol of groups 5 and 6.

The liver weights were greater for groups 1 and 2 than for groups 3 and 4 ($P < 0.01$). The liver weights of groups 5 and 6 were almost twice those of the other groups. Increasing the choline had no effect.

The total liver lipids and the liver cholesterol were several times higher ($P < 0.01$) when cholesterol and cholic acid were added to the diets containing the fat mixture (groups 5 and 6) than for any other groups. This observation held when calculated on both milligrams per gram and total liver bases. A significant decrease in the liver cholesterol ($P < 0.02$) was produced by the higher level of choline (group 6). The livers from the animals fed diets containing the fat mixture (groups 3 and 4) were of low lipid content and were practically devoid of cholesterol. The liver lipids and cholesterol for the rats fed egg oil (groups 1 and 2) were much higher on both the milligrams per gram and grams per liver bases ($P < 0.01$) than for the corresponding groups given the fat mixture (groups 3 and 4). In the egg oil groups, the increased choline produced a significant decrease in the total lipids ($P < 0.02$), but not in the cholesterol.

Experiment 2. The food consumption averaged the same for all groups — 14 g/day. The rats fed the diets containing cholesterol were somewhat heavier than were those fed cholic acid. One rat in each of the groups receiving cholesterol (groups 7 and 8) had a bloody nose and rough fur. All animals fed the like diet but with cholic acid appeared normal. Data for experiment 2 are given in table 3.

The plasma cholesterol showed almost a threefold increase when cholesterol was

[3] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.
[4] Dow Chemical Company, Midland, Michigan.

TABLE 2

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg/100 ml*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 1, 10 weeks [1]							
1 Egg oil; 0.1% choline	312	102 ± 2.4 [2]	11.9 ± 0.75	207 ± 11.5	2.50 ± 0.19	43.4 ± 2.3	0.48 ± 0.05
2 Egg oil; 0.3% choline	298	104 ± 4.3	11.3 ± 0.35	166 ± 8.5	1.95 ± 0.16	40.6 ± 2.4	0.47 ± 0.04
3 Fat mixture; 0.1% choline	301	88 ± 7.5	8.9 ± 0.35	66 ± 3.8	0.57 ± 0.03	3.1 ± 0.3	0.03 ± 0.00
4 Fat mixture; 0.3% choline	286	66 ± 4.8	7.8 ± 0.71	64 ± 2.0	0.51 ± 0.02	4.2 ± 0.4	0.03 ± 0.00
5 Fat mixture; 0.1% choline cholesterol; cholic acid	292	471 ± 60.9	20.6 ± 0.71	355 ± 9.0	7.30 ± 0.37	164.4 ± 5.4	3.57 ± 0.13
6 Fat mixture; 0.3% choline cholesterol; cholic acid	290	547 ± 57.6	20.0 ± 0.71	344 ± 9.8	6.90 ± 0.40	143.6 ± 4.8	2.66 ± 0.20

[1] Each group consisted of 10 female rats with an average initial weight of 214 g.
[2] SD of mean.

TABLE 3

Effects of cholesterol, cholic acid and choline on the weights and lipids of rats fed egg oil or a fat mixture

	Weights	Plasma	Liver				
	Final	Cholesterol	Weight	Total lipids		Cholesterol	
	g	*mg %*	*g*	*mg/g*	*g/liver*	*mg/g*	*g/liver*
Experiment 2, 8 weeks [1]							
7 Fat mixture; 0.1% choline cholesterol	302	273 ± 9.5 [2]	9.7 ± 0.72	329 ± 0.7	3.43 ± 0.02	113.9 ± 5.9	1.19 ± 0.01
8 Fat mixture; 0.3% choline cholesterol	288	297 ± 32.2	13.0 ± 1.6	294 ± 4.5	4.26 ± 0.36	63.9 ± 17.3	0.77 ± 0.05
9 Fat mixture; 0.1% choline cholic acid	265	95 ± 8.7	9.3 ± 0.14	120 ± 17.5	1.10 ± 0.04	17.6 ± 4.5	0.16 ± 0.02
10 Fat mixture; 0.3% choline cholic acid	272	103 ± 15.2	9.7 ± 0.61	91 ± 11.5	0.86 ± 0.06	13.0 ± 4.6	0.12 ± 0.01
Experiment 3, 5 weeks [3]							
11 Egg oil; 0.3% choline cholic acid	264	209 ± 31.3	16.3 ± 0.46	185 ± 7.5	3.02 ± 0.17	50.3 ± 1.9	0.82 ± 0.03
12 Egg oil; 0.3% choline	287	123 ± 8.8	14.1 ± 0.76	170 ± 8.5	2.38 ± 0.14	25.3 ± 5.5	0.39 ± 0.04

[1] Each group consisted of 3 female rats with an average initial weight of 240 g.
[2] SD of mean.
[3] Each group consisted of 5 female rats with an average initial weight of 214 g.

in the rations (groups 7 and 8) than when cholic acid-containing diets were fed (groups 9 and 10), ($P < 0.01$). The plasma cholesterol was not affected by the choline intakes.

The liver weights appeared to he increased by the higher level of choline in the presence of cholesterol (groups 7 and 8); however, this increment was not significant because of the variance and small number of animals in these groups. Increased choline with cholic acid was without effect (groups 9 and 10).

The total liver lipids and cholesterol followed the same pattern as did the plasma cholesterol in that a threefold increase was noted for the rats fed cholesterol (groups 7 and 8) over those receiving cholic acid (groups 9 and 10). The values for these latter groups (groups 9 and 10) were lower than for any other groups in experiments 1 and 2 except those receiving only the fat mixture (groups 3 and 4). Increasing the choline in the presence of cholesterol produced a significant rise ($P < 0.05$) in the lipids on a gram per liver basis, but not on a milligram per gram basis (groups 7 and 8). The cholesterol did not follow this pattern on a total weight basis; in fact, a lower value was noted. Due to the variance in the small number of observations for groups 9 and 10 the choline appeared to be without significance.

The fat mixture with cholesterol produced a two- to threefold increase in plasma and liver cholesterol and liver total lipids over that observed with the egg oil in experiment 1. The modest increase in food intakes in experiment 2 would not appear to account for this difference.

Experiment 3. The food consumption for the 2 groups in this experiment was higher than in experiment 1 in which egg oil was fed even though the initial weights were the same; the food intake being approximately 20 g/day as compared with 12 g in experiment 1. The weight gains were somewhat less for the animals receiving cholic acid than for those without it (groups 11 and 12). Both groups were in good condition throughout the 5-week experiment. The data for experiment 3 are shown in table 3.

Cholic acid produced an increase of approximately 50% in plasma ($P < 0.05$) and liver cholesterol ($P < 0.01$) and a 20% increment in liver lipids based on grams per liver ($P < 0.02$) over the group not receiving cholic acid. These observations suggest the need for a study of egg lipids as influenced by cholic acid in other species including man.

HISTOLOGY — (Experiments 1 and 2)

The livers from the animals receiving cholesterol and cholic acid (groups 5 and 6) were brownish yellow in color and had a butter-like consistency.

Histological examination revealed that 7 out of the 10 animals receiving the diets containing egg oil (groups 1 and 2) had needle-like crystals, presumably cholesterol, in the cytoplasm. The nuclei of the peri-central zone was atrophic and the cells appeared somewhat atrophied. Increasing the level of choline (group 2) appeared to have little or no effect upon the livers. The values for the liver cholesterol were the same for these groups, an observation noted in experiment 1. Rats that were fed cholesterol and cholic acid with the fat mixture (groups 5 and 6) showed massive fatty depositions with innumerable crystals in the parenchymal cells. The liver cords were somewhat distorted due to the great increase in cell size; in fact, the cell appeared to be completely replaced by fat with little evidence of cytoplasmic remnants. The nuclei were atrophic. Increasing the choline in the diet appeared to have little effect, although slightly less lipid and crystalline deposition was noted.

When cholesterol alone was given in the diet containing 0.1% choline (group 7) a massive crystalline deposition with atrophy of the nuclei was noted. These changes were not as marked as in group 8 in which 0.3% choline was given. The livers of the rats fed the fat mixture without cholesterol and cholic acid (groups 3 and 4) showed only mild fatty deposits which were primarily periportal. Increasing the choline was without effect upon these livers. When cholic acid was given with the fat mixture with either level of choline (groups 9 and 10) the fatty deposits were the least observed for any of

the groups. There were few crystals and only a small number of periportal hepatic parenchymal cells. The choline level was without effect.

The livers from experiment 3 were not examined.

The feeding of egg oil with or without cholic acid resulted in lower plasma and liver cholesterol values in rats than did a mixture of fats with the same level of cholesterol present in egg oil. The results appear to indicate that factors, or a balance of factors, present in egg oil tended to lower the cholesterol levels that would have been expected from the feeding of cholesterol as contained in the egg oil. This observation may hold only for the Wistar rat and would not necessarily be in conflict with the observations of Messinger et al. (11) and Bronte-Stewart et al. (5) who used human subjects. Furthermore, egg oil was fed in our experiments; Messinger used egg yolk powder and Bronte-Stewart fed fresh eggs.

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Importance of Dietary Copper in the Formation of Aortic Elastin[1,2]

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ABSTRACT The elastin content of the aortas of newly hatched chicks is approximately 5% of the wet weight of the aorta. When chicks were fed a diet containing 25 ppm copper, the elastin content increased to 12% by the seventeenth day. When the diet contained less than 1 ppm copper, the elastin content of the aorta increased more slowly and never equalled that of the control chicks. The addition of copper to 27-day-old copper-deficient chicks resulted in an increase in aortic elastin concentration to that of the control chicks by the sixteenth day of supplementation. Radioisotope studies, using valine-1-C^{14} to study the metabolism of elastin, revealed that this protein is relatively inert once formed. The results of these studies suggest that the lesion in copper deficiency affected the synthesis of elastin. Amino acid analysis of elastin from copper-deficient and control chicks revealed that the lysine concentration of the copper-deficient elastin was 3 times that of control elastin.

Copper deficiency in chickens and swine results in mortality associated with rupture of the aorta (1, 2). Histological studies of this vessel indicate a derangement of connective tissue, primarily elastin. Such studies suggested a degeneration of elastic tissue. This histological evidence was reinforced by the observations of Kimball et al.[3] on the quantitative reduction in elastin content of the aortas of copper-deficient swine.

The studies presented in this report were designed to 1) measure the reduction in aortic elastin from copper deficient chickens; 2) investigate some aspects of copper deficiency on elastin synthesis; and 3) determine the effect of copper deficiency on the amino acid content of aortic elastin.

EXPERIMENTAL

White Plymouth Rock chicks, obtained from a commercial hatchery, were housed in electrically heated battery brooders and fed the experimental diets ad libitum from the day of hatching. The basal diet used throughout these studies (table 1) was analyzed for copper by the method of Parks et al. (3) and was found to contain 0.8 ppm. The drinking water was demineralized by passing it through a standard bed ion exchange resin[4] and fed in stainless steel troughs. Cupric sulfate was added to supply 25 ppm copper in the control diet.

TABLE 1

Basal diet

	%
Glucose [1]	32.5
Dried skim milk	60.0
Vegetable oil [2]	5.0
DL-Methionine	0.3
L-Arginine·HCl	0.5
Glycine	0.5
NaCl (reagent grade)	0.5
	mg/kg
Vitamin mixture [3]	1980
Choline chloride	1540
$FeSO_4 \cdot 7H_2O$	528
$MnSO_4 \cdot H_2O$ (reagent grade)	220

[1] Cerelose, Corn Products Refining Company, New York.
[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[3] Supplied per kg of diet: (in milligrams) thiamine, 3.52; riboflavin, 5.27; folic acid, 1.10; DL-α-tocopheryl acetate, 22.00; menadione sodium bisulfite, 0.792; biotin, 0.176; pantothenic acid, 18.3; niacin, 52.3; pyridoxine, 5.7; and vitamin B_{12}, 8.8 μg; vitamin A, 11,000 USP units; and vitamin D, 1,980 ICU.

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[2] Published with the approval of the Director of the Experiment Station as Paper no. 1696 of the journal series.
[3] Kimball, D. A., W. F. Coulson and W. H. Carnes 1962 Some properties of aortic elastin of copper deficient swine. Federation Proc., *21*: 121 (abstract).
[4] Barnstead Still and Sterilizer Company, Boston, Massachusetts.

For the analysis of elastin a segment of the aorta, including the common carotids to the point of division into the subclavian arteries and the main aorta from the base of the heart to a point on the aortic arch equal in length to the carotids, was removed, cleaned of adhering tissue, blotted and weighed. The aorta was cut into small pieces and placed in 20-ml test tubes with 15 ml of 0.1 N NaOH. The tubes were incubated in a 100°C water bath for 90 minutes after which the supernatant liquid was decanted and the residue washed twice with distilled water. The insoluble residue, elastin, was dried in a vacuum oven at 50°C overnight, and the amount of elastin determined by weighing the dried material. This is essentially the method of McGavock and Kao (4). Elastin content is expressed as the percentage dry weight of elastin of the wet weight of the aortic segment.

In the radioactive isotope studies, chickens were injected intraperitoneally with 10 μc of valine-1-C^{14}. The aortas were removed at various intervals after injection and the elastin prepared as described. The elastin was then solubilized by treating with 88% formic acid at 100°C for 4 to 5 hours. The material was then added to 15 ml of scintillator mixture[5] and the radioactivity was measured in a Tri Carb liquid scintillation counter. The activity is expressed as counts per minute per milligram of elastin.

The amino acid analysis was conducted in the laboratories of Analytica Corporation, New York, using an automatic amino acid analyzer. For this purpose elastin was prepared from the aortas of five 14-day-old chicks that had been fed the copper-deficient diet, as well as from 5 that had been fed the control diet. For comparative purposes elastin prepared from 5 one-day-old chicks was also prepared. The elastin samples were pooled according to the 3 groups and a single analysis was conducted on each pooled sample.

RESULTS AND DISCUSSION

The results from 2 experiments on the elastin content of the aortas from chickens fed the copper-deficient and control diets are presented in table 2. Since preliminary studies indicated that the moisture and fat content of control and copper-deficient aortas were not different, the results are expressed relative to the wet weight of the aortas. The data showed that when the chicken is hatched, the elastin content of the aorta is approximately 5%. When the chickens were fed the copper-containing diet, the elastin content of the aorta increased rapidly to a value of approximately

[5] The scintillation mixture consisted of 540 ml toluene, 375 ml absolute alcohol, 60 ml of scintillator (9.6 g 2,5,diphenyloxazole plus 36 mg 1,4-bis-2-(4-methyl-5-phenyloxazoly)-benzene in 240 ml of toluene), and 50 g of thixotropic gel powder (Cab-O-Sil Gel, Packard Instrument Company, La Grange, Illinois).

TABLE 2

Effect of copper deficiency on aortic elastin

Age	Experiment 1		Experiment 2	
	Elastin [1]		Elastin	
	Control	Cu-deficient	Control	Cu-deficient
days	%	%	%	%
1 [2]	5.5 ± 0.3	—	4.8 ± 0.4	—
3 [3]	6.5 ± 0.3	6.7 ± 0.5	6.5 ± 0.0	5.5 ± 0.3
7 [4]	9.3 ± 0.0	7.3 ± 0.7	8.6 ± 0.4	6.5 ± 0.6
9 [5]	8.9 ± 0.2	5.4 ± 0.4	—	—
14 [5]	9.3 ± 0.7	6.4 ± 0.4	10.1 ± 0.9	6.6 ± 0.3
17 [5]	12.6 ± 0.2	7.1 ± 0.7	—	—
21 [5]	11.1 ± 0.3	7.0 ± 0.2	12.1 ± 0.6	8.7 ± 0.8
28 [5]	12.1 ± 0.6	8.9 ± 0.7	—	—
49 [5]	12.0 ± 0.4	8.1 ± 0.5	—	—

[1] Elastin is expressed as percentage of the wet weight of the aorta.
[2] Experiments 1 and 2 are the mean values of 2 groups of 5 pooled aortas with SE.
[3] Experiment 1 is the mean value of 4 groups of 5 pooled aortas; experiment 2 is the mean value of 2 groups of 3 pooled aortas.
[4] Experiments 1 and 2 are the mean values of 2 groups of 3 pooled aortas.
[5] Experiments 1 and 2 are the mean values of 5 individual aortas.

12% at 17 days of age. On the other hand, when the diet was deficient in copper, the elastin content increased more slowly and never equalled that of the control chickens up to 49 days.

To determine whether this decrease in aorta elasin content in copper deficiency could be reversed by addition of copper to the diet, a third experiment was conducted. In this experiment the chickens had been fed a copper-deficient diet for 27 days. At this time a sample group of 5 chickens were killed and the elastin content of the aorta was determined. The remaining chickens were divided into 2 lots of equal average weights. One group continued to receive the basal diet, and the other received the basal diet supplemented with 25 ppm copper. After 8 and 16 days, chicks from both groups were killed and the elastin content of the aorta determined. These values were compared throughout the experiment with those obtained from chickens receiving the copper-supplemented control diet from the day of hatching. The results presented in table 3 indicate that after 16 days of copper supplementation the elastin content of the aortas of copper-deficient chicks had returned to normal.

The results of these experiments indicate that copper plays some role in the metabolism of elastin in the aorta. Whether this role is anabolic or catabolic, however, is unknown. To obtain further insight into this aspect of the problem, use was made of radioactive isotope techniques. In these experiments valine-1-C^{14} was used since it has been reported that elastin has a relatively high concentration of this amino acid (5). The results of the first 2 experiments are presented in table 4. In the first experiment the chicks were 43 days of age and in the second, 21 days. As far as could be determined from these data the rate of incorporation of valine into the aortic elastin was the same in both groups. The slightly higher values observed in elastin from deficient birds were probably the result of the deficient chicks being smaller than the control chicks. In neither dietary group did the radioactivity fall appreciably after reaching maximal values, indicating that this protein is relatively inert. This would suggest that the catabolism of elastin was not measurably affected by dietary copper.

To determine whether copper deficiency affected the synthesis of elastin in the very early stages of growth, two more isotope studies were conducted using valine-1-C^{14}. In one, the chickens were 2 days old and in the other, 3 days. The results of that study are presented in table 5. Rapid labeling of the elastin occurred in both the

TABLE 3

Effect of copper supplementation on aortic elastin

Age	Elastin [1]		
	Control	Cu-deficient + Cu [2]	Cu-deficient
days	%	%	%
27	11.1 ± 0.5 [3]	—	7.0 ± 0.3
35	11.3 ± 0.6	9.1 ± 0.4	7.6 ± 0.6
43	11.1 ± 0.6	11.0 ± 0.2	8.4 ± 1.0

[1] Elastin is expressed as percentage of the wet weight of the aorta.
[2] After 16 days of feeding Cu supplement to Cu-deficient chicks.
[3] Mean values of 5 individual aortas with SE.

TABLE 4

Specific activity of elastin from chickens injected with valine-C^{14}

Hours after injection	Specific activity [1]			
	43 days of age		21 days of age	
	Control	Cu-deficient	Control	Cu-deficient
3	4 [2]	11	34	33
6	6	18	55	66
12	10	10	78	71
24	6	19	67	96
48	9	14	78	102
72	12	13	81	114
96	16	26	78	107
120	19	24	—	—

[1] Specific activity expressed as count/min/mg elastin.
[2] Mean value of elastin from 2 aortas.

control and the deficient chicks, but the total activity incorporated was much lower in the elastin from the deficient chicks. These data suggest that the presence of copper is most critical during periods of rapid elastin synthesis, provided that both the normal and deficient aortas contained the same concentration of valine.

To establish this point and to determine the amino acid composition of the elastin from the control and copper-deficient chicks, an amino acid analysis of elastin from the 2 sources was carried out. In this experiment elastin prepared from chicks fed the control and copper-deficient diets for 14 days was used. In addition elastin was prepared from one-day-old chicks for comparative purposes. The results of that analysis are presented in table 6.

The valine content of the control and copper-deficient elastins was not appreciably different. This observation, coupled with the isotope experiment using the 2- and 3-day-old chicks, suggests that copper plays a role in synthesis of normal elastin.

The elastin from the copper-deficient chicks was found to have a much higher lysine content. This has also been observed by Weissman in copper-deficient swine.[6]

[6] Dr. Norman Weissman, University of Utah School of Medicine, personal communication.

TABLE 5

Specific activity of elastin from 2- and 3-day-old chickens, injected with valine-C^{14}

Minutes after injection	Specific activity			
	2 days of age		3 days of age	
	Control	Cu-deficient	Control	Cu-deficient
	counts/min/mg elastin		*counts/min/mg elastin*	
30	389 [1]	149	150	64
60	448	312	513	317
120	596	201	652	255
180	—	—	654	402

[1] Values are from 5 pooled aortas.

TABLE 6

Amino acid analysis of chicken aortic elastin [1,2]

	1-day-old chick elastin	14-day-old chick elastin	14-day-old Cu-deficient chick elastin
	g/100 g	*g/100 g*	*g/100 g*
Hydroxyproline	2.67	1.77	1.90
Aspartic acid	0.30	0.23	0.25
Threonine	0.71	0.84	0.78
Serine	0.45	0.51	0.48
Glutamic acid	1.60	1.64	1.81
Proline	12.31	15.65	15.05
Glycine	26.04	24.42	25.79
Alanine	14.74	14.50	14.44
½ Cystine	trace	trace	trace
Valine	18.23	19.50	18.82
Methionine	trace	trace	trace
Isoleucine	2.32	2.60	2.12
Leucine	7.41	6.94	6.92
Tyrosine	2.24	2.10	2.08
Phenylalanine	3.19	2.97	2.83
Hexosamine	present	present	present
Hydroxylysine	present	present	present
Lysine	0.85	0.83	2.66
Histidine	0.00	0.00	0.00
Ammonia	0.29	0.27	0.28
Arginine	0.74	0.76	1.08

[1] The amino acid analysis was conducted in the laboratories of Analytica Corporation, New York, using an automatic amino acid analyzer.
[2] Values are from a composite sample of elastin prepared from 5 aortas in each group.

This appears to be a rare instance, then, of a nutritional deficiency resulting not only in lowered protein synthesis, but also in the formation of a different protein from that formed in the absence of the deficiency. During the course of these investigations it was observed that elastin from copper-deficient chicks invariably solubilized more quickly in formic acid than did elastin from control chicks. It is possible that the difference in lysine content reflects some basic differences in the configuration and internal binding of the elastin protein which is reflected in the differences in solubility.

One other point should be made. Copper deficiency reduces the growth rate of the chicks. Examples of weights of chicks fed the control and copper-deficient diets are presented in table 7. The weights up to 21 days are from the same experiment, and the 28- and 49-day weights are from two other experiments. The results indicate that 21-day-old copper-deficient chicks weigh on the average approximately as much as 14-day-old control chicks. The data presented in table 2 reveal that the aortas from 21-day-old deficient chicks contain *less* elastin than those from 14-day-old control chicks. The effect of copper deficiency on aortic elastin, therefore, is *not* due to some nonspecific decrease in the rate of development of the whole animal.

TABLE 7

Effect of copper deficiency on body weight of chicks

Age	Average body weight	
	Cu-deficient	Control
days	*g*	*g*
1	44	44
7	81	90
14	128	147
21	158	242
28	204	283
49	506	554

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Dietary Fat and the Structure and Properties of Rat Erythrocytes[1]

II. STABILITY OF THE ERYTHROCYTE

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ABSTRACT The influences of dietary fat and vitamin E deficiency on the stability of rat erythrocytes was investigated. The major changes arose as a result of the omission of vitamin E from the diet. These changes were more pronounced when corn oil was included in the diet. Erythrocytes from vitamin E-deficient animals were susceptible to hemolysis by copper and iron. Those from the supplemented animals were hemolyzed by copper but not by iron. In this case, the degree of hemolysis was less than that noted in the deficient groups. The action of iron appeared to be associated with the presence of oxyhemoglobin in the cell. A hypothesis for this activity is presented and the relationship to the nature of the dietary fat is discussed.

The response of erythrocyte lipids to dietary factors has been demonstrated by numerous investigators (1–3). Since the lipids are involved in the surface structure of the cell, it is feasible that dietary factors may exert some influence on the integrity of erythrocytes subjected to adverse conditions. The action of vitamin E in preventing oxidative hemolysis of the red cell by dialuric acid or hydrogen peroxide is a well known example of the role of nutrients in this respect (4). A reduction in the erythrocyte survival time has also been noted in cases of vitamin E deficiency (5, 6). In the present study, the influence of vitamine E and dietary fat on metal induced hemolysis was studied.

MATERIALS AND METHODS

Nutrition. Four groups of weanling male albino rats were fed for 4 months a diet consisting of glucose, 68%; casein, 18%; inorganic salt mixture, 4%; fat, 10% and water-soluble vitamins (7). The fat-soluble vitamins were administered in stripped corn oil by dropper once each week. The first group was fed 10% corn oil + vitamin E; the second, 10% corn oil, no vitamin E; the third, 10% lard + vitamin E; and the fourth, 10% lard, no vitamin E. The corn oil and lard had been stripped of tocopherols by vacuum distillation.[2] The animals were fed ad libitum and were weighed weekly.

Red cell count. Red cells were enumerated in a counting chamber with the Improved Neubauer ruling. The procedure outlined by Wintrobe (8) was followed.

Oxidative hemolysis. A blood sample was taken from the tail of the rat and subjected to the dialuric acid hemolysis test for vitamin E deficiency proposed by Friedman et al. (9).

Osmotic hemolysis. Five-tenths of a milliliter of blood was withdrawn from a fresh cut in the animal's tail and suspended in 1.0% saline solution. The cells were separated by centrifugation, washed twice with saline, and 0.1 ml of the packed red cells was suspended in 12 ml of 1.0% saline. One milliliter of this suspension was pipetted to each of a series of tubes containing sodium chloride solution, the final salt concentrations ranging from 0.1 to 1.0%. After incubation for one hour at 25°C, the cells were removed by centrifugation and the optical density of the supernatant hemoglobin solution was determined at 415 mμ. The degree of hemolysis was found as in the procedure cited above for oxidative hemolysis, the 0.1 and 1.0% saline solutions representing 100%

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[2] The stripped fats were donated by Distillation Products Industries, Rochester, New York.

and 0% hemolysis, respectively. The degree of hemolysis was plotted as a function of the sodium chloride concentration, and the concentration at which 50% hemolysis occurred was ascertained and used as a basis for comparing the osmotic fragilities of erythrocytes from the various groups.

Metal induced hemolysis.[3] Blood was withdrawn and the red cell suspension prepared as for the osmotic hemolysis experiment, but 0.9% sodium chloride was used. In all instances, 1.0 ml of this suspension was incubated with 9.0 ml of the experimental solution which always had a final sodium chloride content of 0.9%.

In an initial experiment, the cells were incubated with 5.0 μg of each of the following metal ions: manganese (as $MnCl_2 \cdot 2H_2O$), iron (as $FeSO_4 \cdot 7H_2O$), zinc (as $ZnSO_4 \cdot 7H_2O$) and cadmium (as $CdCl_2$). The degree of hemolysis was determined for each metal. Iron and copper were subjected to further examination, the effects of metal concentration and time on the degree of hemolysis being studied.

The effect of replacing cellular oxygen by carbon monoxide was also considered in the case of iron and copper. After drawing the blood, cell suspensions were exposed to carbon monoxide or oxygen, by replacing the atmosphere above the suspensions with the appropriate gas and gently inverting the tube 10 times. This was repeated four more times, and the cells were then washed and resuspended in saline as before. Replacement of cellular gas was effected at zero, 30 and 60 minutes after drawing the blood from the animal. Incubation and hemolysis determination were carried out as before.

Lipid peroxide determination. The procedure described by Tsen and Collier (10) for the detection of lipid peroxides in erythrocyte hemolysates by the use of the 2-thiobarbituric acid test was followed.

Protective action of tocopherol. An emulsion of α-tocopherol in 0.9% sodium chloride was prepared with the aid of polyoxyethylene sorbitan monooleate (Tween 60). The emulsion was then added to the incubation mixture to give final concentrations of 1.0 and 2.0 μg of the vitamin. Hemolysis was determined as before. Blank analyses were carried out using the emulsifier and metal without any tocopherol, and the emulsifier alone.

RESULTS

Differences in the mean weight gains between the groups that had received the vitamin E supplements and those deprived of the vitamin were 81 g and 24 g for the corn oil and lard diets respectively (table 1). These differences were significant at the 0.1% level or below in both cases, as ascertained by the *t* test of significance (11). Moreover, lard was more effective in promoting the growth of the animals than was corn oil. This was so when vitamin E was supplied (groups 1 and 3) or withheld (groups 2 and 4). The difference between groups 1 and 3 was significant at the 0.5% level, that between groups 2 and 4 at less than the 0.1% level. The fur of the animals fed the supplemented diets was smooth and sleek, but that of the deficient rats was coarse and had a reddish cast. There was also visible evidence of testicular degeneration (decrease in size) in the deficient animals.

Oxidative hemolysis, induced by dialuric acid, indicated that the animals that

[3] The metals were studied as their cations. For simplicity the terms "metal" and "metal-induced" are used throughout this paper, rather than the "metal-ion" designation.

TABLE 1

Relationship of the dietary fat to growth and the red cell counts of the experimental animals

Group		Weight gain [1]	Red cell count [2]	Range [3]
		g		
1	Corn oil + vitamin E	324 ± 21	8.40 ± 0.36	7.98 – 8.80
2	Corn oil, no vitamin E	241 ± 16	7.62 ± 0.79	6.75 – 8.80
3	Lard + vitamin E	340 ± 23	8.97 ± 0.50	8.11 – 9.32
4	Lard, no vitamin E	316 ± 20	8.91 ± 0.20	8.59 – 9.12

[1] Mean weight gain of 45 animals ± SD of mean.
[2] Mean count for 5 animals ± SD in millions of cells/mm³.
[3] Millions of cells/mm³.

had received the tocopherol supplements (groups 1 and 3) were not deficient, whereas those that had not received the supplements (2 and 3) were deficient. Groups 1 and 3 had mean hemolysis values of 1.0 and 0.0%, respectively, and groups 2 and 4 had values of 99 and 96%, respectively.

The red cell counts of the animals in the four groups are presented in table 1. The differences between the groups were not significant as determined by the *t* test. However, the range of values obtained with the rats from group 2 (corn oil, no vitamin E) was considerably greater than those obtained for the other groups. The minimal value for this group may indicate the onset of anemia in individual rats within this group.

The saline concentrations at which 50% hemolysis occurred were 0.48, 0.48, 0.50 and 0.50% for groups 1, 2, 3, and 4, respectively. The differences were of doubtful significance.

Of the metals examined, only iron and copper were found to induce significant hemolysis (greater than 10%) when used at a concentration of 5 μg in 10 ml of the incubation mixture. Copper was effective in hemolyzing cells from all 4 groups but was slightly more effective with the cells from the deficient animals. Iron induced considerable hemolysis when incubated with cells from the deficient animals but was essentially without effect (2 to 3% hemolysis) when cells from groups 1 and 3 were used. Figure 1 represents the variation in hemolysis with copper concentration for cells from the four groups of animals. Figure 2 represents the effect of iron on the degree of hemolysis of cells from animals deficient in vitamin E. The incubation time in the case of copper was 60 minutes. A 40-minute incubation was used in the experiments with iron since the longer incubation period obscured important differences between the 2 groups studied. This is evident from figure 3, showing the relationship of the incubation time to the degree of hemolysis. A similar experiment was conducted for the incubation of copper with the erythrocytes from the animals in the deficient groups, but no difference was observed in this case.

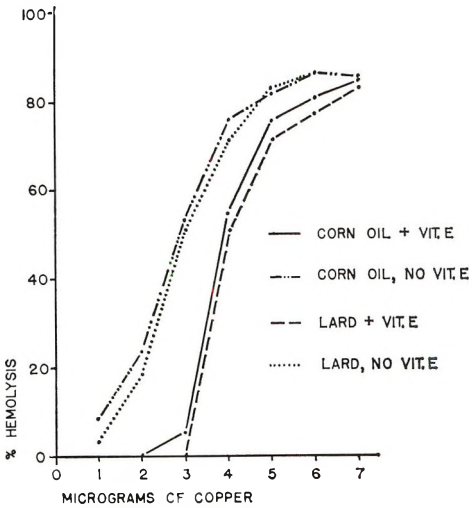

Fig. 1 Variation in hemolysis with copper concentration. Conditions: 60 minutes' incubation at 25°C.

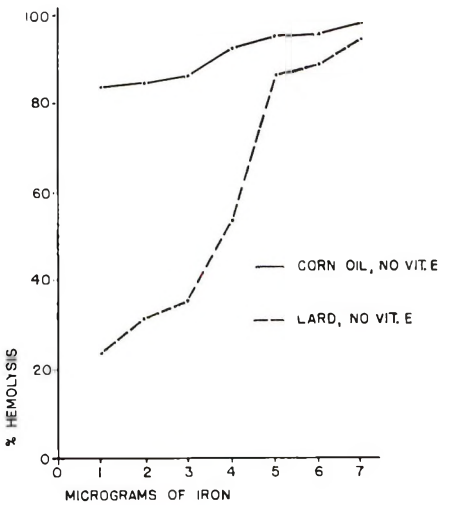

Fig. 2 Variation in hemolysis with iron concentration. Conditions: 40 minutes' incubation at 25°C.

The replacement of cellular oxygen by carbon monoxide greatly influenced the degree of metal-induced hemolysis (table 2). Allowing the cells to stand at room temperature prior to replacement of the oxygen also had a marked effect on the hemolysis. In the case of iron, the presence of carbon monoxide afforded some measure of protection irrespective of the time elapsed before replacement of the

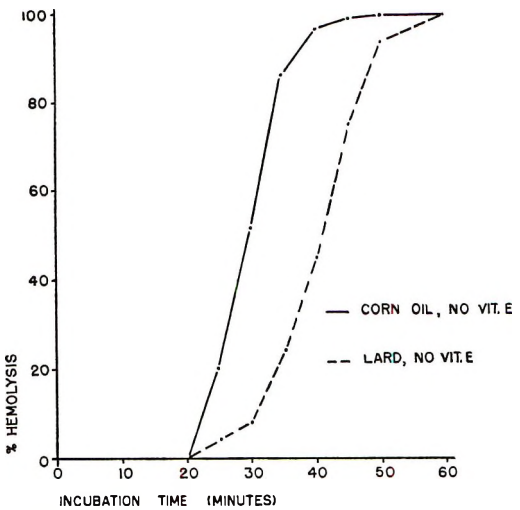

Fig. 3 Variation in iron induced hemolysis with time. Conditions: Incubation with 4 μg of iron at 25°C.

TABLE 2

Effect of replacement of cellular oxygen by carbon monoxide [1]

Time [2]	Gas	Copper,[3] % hemolysis	Iron,[3] % hemolysis
0	Oxygen	25.9	1.2
	Carbon monoxide	8.2	1.0
30	Oxygen	33.3	15.7
	Carbon monoxide	47.4	4.4
60	Oxygen	29.2	51.4
	Carbon monoxide	38.9	18.8

[1] Metal concentration 4 μg, incubation time 40 minutes. Animals from group 4, lard, no vitamin E used.
[2] Time in minutes elapsed between drawing, washing and centrifuging the cells, and replacing the oxygen with carbon monoxide and commencing the incubation with the appropriate metal.
[3] Values represent the mean of 3 animals.

oxygen. However, the elimination of oxygen inhibited copper hemolysis only if carried out immediately after blood was drawn. After the cells had been in contact with oxygen for some time, there was no inhibition but an increase in hemolysis.

DISCUSSION

Variation of the dietary fat and the omission of vitamin E from the diet resulted in changes in the stability of erythrocytes. Vitamin E deficiency resulted in the most significant changes, whereas the nature of the dietary fat tended to modify the degree of change.

Reduction in the red cell survival time accompanied by anemia has been observed in the rat (6) and the monkey (5) in cases of vitamin E deficiency. A reduction in the erythrocyte survival time without the symptoms of anemia has been observed in vitamin E-depleted human subjects (12). In the present experiment, deprivation of vitamin E for 4 months did not give rise to a significant level of anemia. The red blood cell counts were inconclusive. Differences were noted between animals receiving lard and corn oil, and between those deficient in vitamin E and receiving the vitamin supplement. These differences were, however, of doubtful significance. The wide range of values obtained with the rats receiving corn oil and no vitamin E may indicate the onset of anemia among individuals within this group.

The role of metals in the oxidative hemolysis of erythrocytes was investigated by Bunyan and his associates (13), who found that copper, zinc, and a number of other metals were antagonistic to the protective action of tocopherol against dialuric acid-induced hemolysis. Iron and nickel were without effect. However, copper had previously been shown to be hemolytic (14). The metal was believed to exert its action by binding to the erythrocyte surface as a protein-copper complex. Vitamin E deficiency was not involved and no other oxidizing agent was present. This copper-induced hemolysis was noted in the present study. It was essentially independent of the dietary fat consumed but was enhanced by vitamin E deficiency, particularly at lower concentrations of the metal ion.

In the case of iron, a sharp difference was noted between the supplemented and deficient animals. Little or no hemolysis occurred in the presence of 5 μg of iron if vitamin E was included in the diet. In the absence of the vitamin, considerable hemolysis occurred. Initial experiments involved a one-hour incubation, and the observed difference between the groups receiving corn oil and lard was small. However, a time study revealed that the dietary fat exerted a considerable influence on the rate of hemolysis (fig. 3). The more unsaturated diet was accompanied

by a shorter induction period. Cells from the animals fed the corn oil diet were susceptible to much lower concentrations of iron (fig. 2). A series of experiments conducted at 25°C indicated that the iron-induced hemolysis was temperature-dependent. The degree of hemolysis at this temperature was considerably lower than at 37°C.

By analogy with the dialuric acid hemolysis system, it appeared possible that oxidative hemolysis, catalyzed by iron, was occurring. This view was supported by the greater susceptibility of the linoleic acid-rich erythrocytes to the action of the metal. However, attempts to demonstrate the occurrence of lipid peroxides by their reaction with 2-thiobarbituric acid proved unsuccessful. When the total hemolysate was used, a brown color resulted from the interference by hemoglobin. If the stroma were first isolated, the expected pink color was obtained but was of low intensity. This may have been due to the use of too small a cell sample, but at higher cell concentrations, hemolysis was extensive and uncontrolled.

Attempts to study the protective action of tocopherol were likewise unsuccessful. In this case, the emulsifying agent, Tween 60, increased the hemolytic action of the metals, probably by enhancing the binding of the metal to the cell surface. The Tween 60 was not itself hemolytic at the concentration used.

The replacement of oxygen by carbon monoxide had been shown to be effective in reducing the degree of hemolysis induced by dialuric acid (15). The same substitution was therefore made in the present study using cells from the vitamin E-deficient animals and copper or iron. Iron became more effective as a hemolytic agent in the presence of oxygen as the pre-incubation period of the cells in the presence of oxygen was increased. Carbon monoxide exhibited a substantial inhibitory effect on the hemolytic action of this metal. These observations permit the formulation of the following hypothesis concerning the action of iron on the cells. The increasing hemolysis of the cells with increasing pre-incubation with oxyhemoglobin indicates that the latter compound is acting on the cell during this time, possibly initiating an oxidation of the cell lipids. The ability of carbon monoxide to reduce the hemolytic activity of iron indicates that the metal, in the presence of oxyhemoglobin, acts on the cell to produce hemolysis. This latter action may be the completion of the partial oxidation of the lipids initiated in the pre-incubation period. The cells from the animals fed corn oil, containing more linoleic acid (3), would presumably be more susceptible to oxidation and hemolysis.

In the case of copper, the carbon monoxide-hemoglobin samples afforded no protection against hemolysis if any pre-incubation with oxygen was permitted. This would indicate that the copper operates independently of the oxyhemoglobin. This is in keeping wtih Lambin's theory (14) concerning the mode of action on copper on the cell, namely, that it binds the surface of the cell. The increased hemolysis found in vitamin E-deficient animals, when compared with the supplemented ones, may result from oxidation in the former case producing more polar groups in the cell surface and facilitating the binding of the metal. This would account for the reduced hemolysis in the presence of carbon monoxide with no pre-incubation period (table 2). The increase in hemolysis after the replacement of oxygen by carbon monoxide after 30 or 60 minutes' pre-incubation is difficult to explain. However, the mere act of exchanging the 2 gases resulted in some hemolysis (which was eliminated when the cells were washed). It is possible that this exchange weakens the cell in general thus facilitating the action of copper.

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Dietary Fat and the Structure and Properties of Rat Erythrocytes[1]

III. RESPONSE OF ERYTHROCYTE FATTY ACIDS TO VARIOUS DIETARY FATS

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ABSTRACT The response of rat erythrocyte fatty acids to 4 fats of widely differing fatty acid compositions was investigated. The red cell fatty acids were susceptible to dietary influence but in a very specific manner. Short-chain dietary fatty acids, provided by butter and hydrogenated coconut oil, were not incorporated into the erythrocyte lipids to any appreciable extent. The "non-mammalian" hydroxy acid, ricinoleic acid, was not incorporated into the cell when castor oil was included in the diet. This was possibly due, in both cases, to the absence from the plasma of significant levels of the acids in question. Diets low in essential fatty acids resulted in low cellular concentrations of linoleic and arachidonic acids, with a concomitant accumulation of oleic and eicosatrienoic acids. The high dietary linoleic acid provided by corn oil resulted in the accumulation of this acid in the cell at the expense of the oleic acid. Fatty aldehydes, derived from the plasmalogen phosphatides, were also responsive to diet, linoleyl aldehyde increasing with increased dietary linoleate.

We have previously shown that changes in the lipids of the erythrocyte resulting from dietary factors involve only the fatty acid moieties of the lipids (1). Such changes are the result of differences in the unsaturated acids in the dietary fat. In an effort to study the effects of these changes on the permeability of the cells to various solutes, the previous work has been extended by using fats of widely different fatty acid composition.

MATERIALS AND METHODS

The basic diet has been described in a previous publication (1). The dietary fats used, all at a level of 10%, were hydrogenated coconut oil, butterfat, castor oil, and corn oil. Weanling male albino rats were fed the diets ad libitum for 22 weeks.

At the end of this period 3 rats were chosen at random from each dietary group and anesthetized with pentobarbital sodium.[2] Blood was withdrawn from the abdominal aorta into sodium citrate solution. The red cells were obtained by centrifugation and were washed 3 times with isotonic saline. They were then added slowly to 7 volumes of absolute methanol. Fourteen volumes of chloroform were added and the extraction was continued for one hour. After filtration, the residue was re-extracted twice more with chloroform-methanol. The extracts were combined, washed with 0.2 volumes of distilled water (2), dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure at room temperature.

The total lipid sample was methylated and the methyl esters sublimed as described previously (1). Separation of the dimethyl acetals of long-chain aldehydes was accomplished by saponification of the total methylate with 5% methanolic sodium hydroxide for 3 hours at 80°C. The nonsaponifiable fraction, containing the acetals, was extracted with hexane. After acidification of the remaining sodium salts, the fatty acids were extracted and remethylated with methanolic hydrogen chloride. Gas chromatographic analysis of the methyl esters and of the dimethyl acetals was carried out as before (1).

RESULTS

The fatty acid compositions of the erythrocyte lipids from the 4 dietary groups are presented in table 1. The major differ-

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

TABLE 1

Fatty acid and aldehyde composition of the erythrocyte lipids [1]

Identity [2]	Coconut oil	Butter fat	Castor oil	Corn oil
12:0	1.5 ± 0.3 [3]	0.7 ± 0.3	0.4 ± 0.1	0.4 ± 0.1
13:0	trace	trace	trace	trace
14:0	1.8 ± 0.2	1.4 ± 0.1	0.9 ± 0.2	0.6 ± 0.1
15:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0
16:0	22.4 ± 1.4	20.6 ± 1.1	25.2 ± 3.4	24.2 ± 1.8
16:1	2.7 ± 0.1	2.3 ± 0.1	1.7 ± 0.5	0.4 ± 0.0
17:0	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
18:0	14.2 ± 0.8	9.8 ± 0.9	13.5 ± 0.7	13.5 ± 1.2
18:1	15.7 ± 1.2	16.9 ± 0.5	13.5 ± 0.7	8.6 ± 0.2
18:2	2.2 ± 0.6	4.3 ± 0.1	5.3 ± 0.3	11.5 ± 0.7
20:0	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:2	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
20:3	15.0 ± 1.7	5.1 ± 0.8	1.3 ± 0.3	0.1 ± 0.1
20:3	1.0 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	0.5 ± 0.1
20:4	15.4 ± 0.5	24.5 ± 1.0	26.3 ± 1.7	31.0 ± 2.3
Unidentified	trace	trace	trace	0.2 ± 0.1
20:5	1.4 ± 0.1	2.9 ± 0.6	0.3 ± 0.1	0.2 ± 0.0
24:1	1.3 ± 0.4	0.8 ± 0.2	3.0 ± 0.5	1.3 ± 0.5
22:4(?)	1.6 ± 0.4	1.6 ± 0.5	2.3 ± 0.6	3.7 ± 0.2
22:5	1.0 ± 0.3	0.3 ± 0.2	0.7 ± 0.0	1.4 ± 0.2
22:5(?)	0.2 ± 0.1	1.2 ± 0.5	0.7 ± 0.3	0.3 ± 0.1
22:6	1.1 ± 0.2	5.0 ± 0.2	2.7 ± 0.3	0.8 ± 0.1
16:0 al	30.5 ± 1.8	27.3 ± 1.2	27.0 ± 2.2	30.2 ± 1.5
18:0 al	28.8 ± 1.5	26.7 ± 1.7	30.9 ± 1.2	31.7 ± 1.4
18:1 al	29.9 ± 1.6	27.3 ± 1.6	31.0 ± 3.1	21.0 ± 1.2
18:2 al	2.9 ± 0.7	4.9 ± 0.7	3.1 ± 0.4	7.1 ± 0.7
Unidentified [4]	7.9	13.8	8.0	10.0

[1] Expressed as a percentage of the total fatty acids or total fatty aldehydes.
[2] Numbers before and after colon represent carbon chain length and number of double bonds, respectively; al signifies aldehyde.
[3] Mean of 3 analyses ± SD of mean. Trace signifies less than 0.05%.
[4] Non-saponifiable components.

ences were in the oleic, linoleic, eicosatrienoic and eicosatetraenoic acids. The oleic acid decreased as the dietary linoleic acid was increased.[3] There was a concomitant increase in the linoleic acid content of the erythrocyte lipids. The highly saturated coconut oil diet resulted in the accumulation of considerable amounts of eicosatrienoic acids, particularly the 5, 8, 11-isomer, which are characteristic of essential fatty acid deficiency. The increasing amounts of dietary linoleic acid found in the butterfat, castor, and corn oils, respectively, resulted in the accumulation of decreasing amounts of the trienoic acids. This decrease was accompanied by an increase in the arachidonic acid content of the cell. The acid tentatively identified as a docosatetraenoic acid also increased when dietary linoleic acid was increased.

Other changes occurring were in the lauric, myristic and palmitoleic acid concentrations in the cell lipids. These all showed increases as the dietary linoleic acid decreased. In the case of lauric and myristic acid, these changes may also be related to the dietary supply of these acids.

In table 1 are also listed the compositions of the dimethyl acetals obtained from the red cells of animals fed the 4 diets. The major identifiable components were the acetals of palmitic, stearic, oleic, and linoleic aldehydes which constituted approximately 90% of the total aldehydes. Again there appeared to be some response to dietary factors, the linoleyl aldehyde increasing with increased dietary linoleate. The butter group was slightly anomalous in this case. This observation suggests that the fatty acids may act as precursors for the aldehydes in plasmalogen biosynthesis.

[3] Major components of dietary fats (as % total fatty acids): corn oil 16:0, 11.5%; 18:0, 2.8%; 18:1, 30.1%; 18:2, 54.0%; 18:3, 0.5%. Castor oil 16:0, 1.7%; 18:0, 1.5%; 18:1, 4.1%; 18:2, 6.6%; 18:1OH (ricinoleic), 83.6%. Butter fat C-4 to C-12, 10.0%; 14:0, 11.2%; 16:0, 26.3%; 18:0, 13.9%; 18:1, 29.5%; 18:2, 2.2%; 18:3, 1.6%. Coconut oil 10:0, 5.0%; 12:0, 50.8%; 14:0, 19.0%; 16:0, 9.9%; 18:0, 10.4%; 18:1, 2.8%; 18:2, 0.6%; 18:3, 0.4%.

DISCUSSION

The four different dietary fats resulted in considerable differences in the total fatty acid patterns of the erythrocyte lipids. These differences may be attributed primarily to varying essential fatty acid content of the dietary fat. As dietary linoleic acid decreased, the cellular linoleic, and to some extent, arachidonic acids decreased, whereas the eicosatrienoic acids increased.

Although changes may be effected in the red cell fatty acids, they are quite specific changes (table 1). The administration of butterfat, which contained shorter chain acids, did not lead to the incorporation of these acids into the cellular lipids. Although coconut oil contained 50% lauric acid, the erythrocytes from animals receiving this dietary fat contained only 1.5% of this acid. A similar situation was noted with castor oil in the diet. This fat contained over 80% of 12-hydroxy octadeca-9-enoic acid. The retention time of this acid under the chromatographic conditions used was very close to that of the C-22 hexaenoic acid. In this region of the chromatogram, the amount of material eluted from the column was 2.7% of the total methyl esters. After treatment of the methyl esters with an acetylating reagent, very little change was noted in the concentrations of the various methyl esters. Thus, little, if any, hydroxy acid was incorporated into the cell lipids. A probable reason for the failure of the cell to incorporate these various acids may be their absence or low level in the plasma. Spot checks on the plasma lipids of the animals fed the coconut and castor oil failed to indicate any appreciable level of either lauric or ricinoleic acid in the respective plasma samples. It has been shown, however, that there is very little incorporation of C^{14}-labeled lauric acid into the red cell in vitro (3). This would support the contention that the degree of alteration of the erythrocyte lipids may be subject to certain limitations.

The primary changes in the erythrocyte fatty acids were in the oleic and linoleic acids and their probable metabolites. Witting and his co-workers (4) and Monsen and her associates (5) have previously demonstrated that replacement of dietary corn oil by coconut oil resulted in a decrease in linoleic acid and an increase in the oleic acid of the erythrocyte lipids. We have also shown this to be true when lard was substituted for corn oil (1). In none of these cases was any significant change noted in the arachidonic acid of the cell. However, the dietary coconut oil contained 4%, and the lard 10% linoleic acid. This coconut oil diet would result in conditions similar to those used with the castor oil diet in the present experiment, and which resulted in a slightly lower arachidonic acid content in the cell lipids when compared with the corn oil diet. The animals receiving the coconut oil diet exhibited the usual external signs of essential fatty acid deficiency, scaliness of the paws and increased food and water consumption. Mohrhauer and Holman (6) have shown that a dietary deficiency of linoleic acid does indeed influence the arachidonic acid content of the red cell lipids, which was also the case in the present study.

The appearance of eicosatrienoic acids is a sign of essential fatty acid deficiency. In a previous paper of this series (1), an acid having a carbon number of 22.2 was identified as an eicosatrienoic acid. This acid was found to increase slightly when dietary linoleic acid was restricted in the present study. However, a much greater increase was noted in the acid occurring just prior to this on the chromatogram (carbon number 21.9–22.0). This component was unsaturated and on hydrogenation behaved as an eicosenoic acid, a fact that was not evident in the original study (1) owing to the low concentration of this acid and the presence of docosenoic acids which would appear in this region of the chromatogram after hydrogenation. On the basis of the increase in this acid in cases of essential fatty acid deficiency and from retention data in the literature, this acid (carbon number 21.9–22.0) was tentatively identified as an eicosatrienoic acid also. Mohrhauer and Holman (7) isolated the eicosatrienoic acids from the liver lipids of rats deficient in essential fatty acids. They characterized them by degradative ozonolysis as the 5, 8, 11- and 7, 10, 13- isomers the former comprising the larger proportion of this mixture. Retention data available in the literature indicate that the 5, 8, 11- isomer is eluted prior to the 7, 10, 13-

isomer. This would make the present results consistent with these of Mohrhauer and Holman since the isomer, 5, 8, 11, with the lower retention time was present in much higher concentrations when coconut oil was included in the diet.

These eicosatrienoic acids result from the efforts by the organism to synthesize arachidonic acid from the saturated and monoenoic acids in the body. This would account for the appearance of more oleic, and to a lesser extent, palmitoleic acid in the cells of the rats fed coconut oil, in spite of the highly saturated nature of this fat. Oleic and linoleic acids are apparently capable of replacing each other quite readily in the red cell phospholipids (3). In this respect the total C-18 unsaturated acids are relatively constant for all 4 diets. This is also true for the total eicosatriene and tetraene acids.

The acid tentatively identified as a docosatetraene in our previous publication (1) was found to increase with increasing linoleate. This is contary to the observations of Enser and Bartley, who noted that this tetraenoic acid was characteristic of essential fatty acid deficiency (8). Mohrhauer and Holman (7) isolated an acid from liver lipids which they believed to be a docosatetraene. Degradation studies indicated it to be a docosapentaene however. They quoted carbon numbers of 25.2, 25.7 and 26.3 for 4, 7, 10, 13, 16-docosapentaenoic, 7, 10, 13, 16, 19-docosapentaenoic and 4, 7, 10, 13, 16, 19-docosahexaenoic methyl esters, respectively. We believe these to correspond to methyl esters appearing on our chromatograms with carbon numbers of 25.4, 25.9 and 26.6. The tentatively identified methyl docosatetraenoate had a carbon number of 24.9. Final identification would require isolation of this acid and degradative studies, neither of which were possible with the detection system (hydrogen flame) used in the present study. The discrepancy between our results and those of Enser and Bartley (8) probably results from the uncertainty of the identity of the acid in question. Docosahexaenoic acid is believed to be synthesized from linolenic acid (7). It was higher in the cell lipids from the animals fed the diet containing butter, which contained 1.6% linolenic acid.

Since a number of aldehyde dimethyl acetals have retention times similar to those of the more abundant fatty acid methyl esters, it was found expedient to separate the alkali stable acetals from the methyl esters by saponification. In this way, linoleyl aldehyde dimethyl acetal, which is normally obscured by methyl oleate under the conditions used, was detected and determined. Again there was a correlation between the dietary linoleate and the amount of this aldehyde present. Similar trends have been noted in human erythrocytes (9). This indicates that enzymatic reduction of fatty acids may be involved in plasmalogen synthesis. Experiments with radioactive palmitaldehyde, palmitic and stearic acids have indicated that the free aldehyde is not incorporated into plasmalogens but fatty acids are incorporated to a small degree (10).

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Metabolic Studies of an Amino Acid Imbalance in Cold-exposed Rats[1]

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ABSTRACT Young rats fed an amino acid-imbalanced diet based on 6% fibrin, and supplemented with 0.4% DL-methionine and 0.6% DL-phenylalanine had increased activities of liver arginine synthetase and arginase, and increased plasma levels of a number of amino acids. However, the activities and levels of these metabolites were decreased in a comparable group of animals exposed to 7°C. The radioactivity of the expired CO_2, originating from 2 μc each of intraperitoneally administered carbon-labeled methionine, phenylalanine or alanine, indicated that the rats kept at room temperature may not be able to oxidize effectively the 2 imbalancing amino acids to maintain a proper balance in the amino acid pool for the protein synthesis. In contrast, the cold-exposed animals were able to catabolize the excessive levels of methionine and phenylalanine and utilize them for energy purposes. The remaining balanced portion of the amino acid pool was then apparently used for the formation of new tissues, as also indicated by greater gains in body weight. In another experiment it was shown that protein-depleted rats exposed to 3°C for 27 hours readily consumed the imbalanced diet, whereas the food intake of a comparable group of rats kept at 25°C decreased sharply 18 hours after the animals had been given the diets.

It is well established that ingestion of an amino acid-imbalanced diet based on 6% fibrin supplemented with 0.4% DL-methionine and 0.6% DL-phenylalanine leads to a growth depression in young rats (1). However, it was recently reported from this laboratory that young rats chronically exposed to a mild cold stress (7°C) readily consumed this imbalanced diet without any apparent adverse effect on growth, despite the fact that these animals doubled their food consumption (2). The present experiments were conducted in an attempt to elucidate this phenomenon and to further investigate metabolic changes associated with this specific amino acid imbalance. In addition, the effect of acute cold stress (3°C) on the consumption of this imbalanced diet by protein-depleted rats was investigated.

METHODS

Male rats, of the Sprague-Dawley strain, ranging in weight from 140 to 180 g were used in all experiments. All rats were housed in individual wire cages and both the diets and water were given ad libitum.

The basal diet consisted of 6% fibrin, 85% sucrose, 5% corn oil, and 4% USP salt mixture no. XIV. The vitamin mixture supplied 2,000 units of vitamin A, 222 units of vitamin D, and the following in mg per 100 g of diet: α-tocopherol, 11.1; ascorbic acid, 100; inositol, 11.1; choline chloride, 166.5; menadione, 5; *p*-aminobenzoic acid, 11.1; niacin, 10; pyridoxine·HCl, 2.22; riboflavin, 2.22; thiamine·HCl, 2.22; Ca pantothenate, 40.3; also 44 μg of biotin, 200 μg of folic acid and 3 μg of vitamin B_{12}. The imbalanced diet was created by adding 0.4% DL-methionine and 0.6% DL-phenylalanine to the basal diet at the expense of sucrose. The protein-free diet consisted of the basal diet in which the fibrin was replaced with an equal weight of sucrose. The following experiments were conducted.

Experiment 1. A group of rats, maintained at 25°C, was fed the protein-free diet for 11 days until they lost 25% of their initial average body weight. Afterwards one-half of the animals were placed in a cold room held at 3°C, while the other group of rats, (henceforth called the "warm" rats) remained in an animal room maintained at 25°C. Both groups were given the experimental diets, and food con-

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[1] The experiments were conducted according to "Rules Regarding Animal Care" as established by the American Medical Association. (Air Force Regulation 90–4.)

sumption records were taken at short intervals for a total period of 27 hours.

Experiment 2. Young rats, previously fed a commercial laboratory diet, and maintained at 25°C, were divided into 2 groups. One group remained in the animal room at 25°C, while the other group was kept in a cold room at 7°C. Both groups were fed the experimental diets for 21 days.

At the end of the experiment the animals were decapitated and the blood collected in centrifuge tubes containing a drop of heparin solution. The liver was immediately excised and chilled in chipped ice. The following methods were applied for the determination of the selected liver components: total fat, the method of Handler (3); phospholipids, the method of Chen et al. (4); cholesterol, the method of Searcy and Berquist (5); arginase and arginine synthetase, the method of Brown and Cohen (6); glutamic-pyruvic (GPT) and glutamic-oxalacetic (GOT) transaminase, the methods of Wróblewski and LaDue (7) and LaDue et al. (8), respectively; tryptophan pyrrolase (TP), the method of Knox and Auerbach (9); tyrosine α-ketoglutaric transaminase (TKGT), the method of Lin and Knox (10). The enzyme activities are expressed in units per gram of fresh tissue. Plasma amino acids were separated by two-dimensional paper chromatography according to Berry and Cain (11) and the quantitative estimation of the separated amino acids was carried out according to the procedure of Bode (12).

Experiment 3. This experiment was conducted to determine the effect of the imbalance and cold exposure on the oxidation of the 2 imbalancing amino acids themselves, and of alanine, to CO_2. The experimental conditions were similar to those described in experiment 2. After both the cold-exposed and the "warm" animals had been fed the experimental diets for 21 days they were given an intraperitoneal injection of 2 μc each of either DL-methionine-1-C^{14} (specific activity 3.4 mc/mmole), L-phenylalanine-U-C^{14} (specific activity 66 mc/mmole) or L-alanine-U-C^{14} (specific activity 89 mc/mmole). Immediately after injection of the radioactive substrate each animal was placed in an all-glass metabolism cage and the expired CO_2 was collected in a solution of ethanolamine in ethylene glycol monomethyl ether (1:2 v/v) in increments over a 12-hour period. One-milliliter aliquots were added to the scintillation medium, consisting of toluene and ethylene glycol monomethyl ether in a 2:1 ratio (v/v) and 5.5 g/liter of 2,5-diphenyloxazole, and radioactivity was counted in a liquid scintillation counter (13). The CO_2 collection was carried out at the temperatures to which the animals had been exposed, and food and water were available to the animals during the whole collection period.

RESULTS AND DISCUSSION

Results of the first experiment are shown graphically in figure 1. Cold exposure markedly stimulated food intake within one hour after the rats had been placed in the cold room. Both cold-exposed groups consumed their respective diets at approximately the same rapid rate, and in 27 hours the animals consumed 24.8 g of the basal and 25.1 g of the imbalanced diet. In contrast, the food intake of the "warm" rats fed the imbalanced diet began to decrease after 12 hours, and declined sharply 18 hours after the beginning of the feeding period. The rats fed the basal diet maintained, however, a steady food intake throughout the experiment, consuming 17.8 g of the diet, whereas those fed the imbalanced diet ate only 9.3 g. The data demonstrate that a cold stress effectively

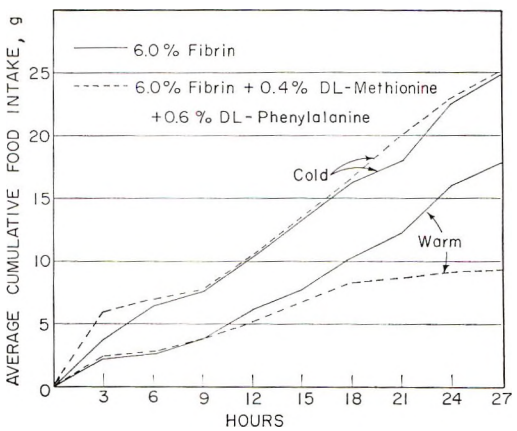

Fig. 1 Effect of amino acid imbalance and cold exposure on food intake of protein-depleted rats. Each point represents the average of 7 animals.

stimulates food intake of protein-depleted rats. A similar effect of cold stress on appetite of non-depleted rats fed amino acid-imbalanced diets was reported previously from this laboratory (2).

Several changes in the levels of certain liver constituents and plasma amino acids associated with the amino acid imbalance and cold exposure are shown in tables 1 and 2, respectively. The activities of arginase and arginine synthetase of both the "warm" and the cold-exposed rats fed the imbalanced diet were increased, when compared with those fed the basal diet (groups 3 and 4 vs. group 1). However, the increase in the activities of these 2 enzymes in the cold-exposed animals was not so great as that of the corresponding

TABLE 1

Effect of cold exposure and amino acid imbalance on selected liver metabolites

	Group and treatment			
	1	2	3	4
	6% Fibrin		6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	
	Warm	Cold	Warm	Cold
Avg Δ body weight, g/3 weeks	40.1 ±2.3 [1]	52.3 ±3.1	31.6 ±2.8	53.6 ±3.9
Avg daily food intake, g	13.4 ±0.62	18.5 ±0.70	10.3 ±0.41	19.1 ±0.21
Liver components				
Total fat, %	5.21±0.12	5.14±0.18	4.98±0.20	5.10±0.14
Neutral fat, %	1.61±0.10	1.70±0.14	1.65±0.15	1.59±0.10
Phospholipids, %	3.41±0.21	3.39±0.18	3.42±0.24	3.50±0.20
Cholesterol, %	0.34±0.04	0.34±0.03	0.35±0.04	0.33±0.02
Arginase, mmole/g/hr	30.1 ±2.9	36.8 ±1.6	44.9*±2.0	37.2 ±1.8
Arginine synthetase, μmole/g/hr	51.4 ±1.6	59.6 ±2.8	64.8*±3.1	55.7 ±2.0
GOT, μmole/g/min	150.2 ±6.3	184.6 ±7.2	156.8 ±7.0	178.6 ±8.5
TKGT, μmole/g/hr	55.3 ±2.4	61.1 ±3.0	57.3 ±2.3	60.8 ±2.8
GPT, μmole/g/min	30.4 ±3.2	39.2 ±2.9	32.1 ±1.8	38.6 ±2.2
TP, μmole/g/hr	2.9 ±0.03	3.1 ±0.08	2.8 ±0.12	2.9 ±0.10

[1] All results are expressed as averages of 5 rats ± SE of mean.
* Difference from group 1 ($P < 0.05$).

TABLE 2

Effect of cold exposure and amino acid imbalance on selected blood plasma components

	Group and treatment			
	1	2	3	4
	6% Fibrin		6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	
	Warm	Cold	Warm	Cold
	mg metabolite/100 ml plasma		*mg metabolite/100 ml plasma*	
Alanine	2.78±0.07 [1]	2.87±0.04	3.34*±0.11	2.74±0.09
Arginine	3.12±0.04	3.18±0.07	3.75*±0.08	3.09±0.08
Aspartic acid	2.23±0.11	2.32±0.14	2.78*±0.09	2.30±0.14
Glutamic acid	3.20±0.15	3.22±0.05	3.98*±0.09	3.38±0.12
Glycine	2.64±0.13	2.52±0.10	2.59 ±0.12	2.51±0.14
Isoleucine	1.38±0.05	1.42±0.09	1.45 ±0.04	1.49±0.09
Leucine	2.10±0.08	2.15±0.09	2.28 ±0.11	2.25±0.10
Lysine	5.35±0.12	5.25±0.16	5.98*±0.15	5.39±0.13
Methionine	1.81±0.13	1.79±0.10	2.36*±0.08	1.88±0.09
Phenylalanine	1.43±0.04	1.32±0.06	2.58*±0.08	1.54±0.12
Proline	3.81±0.12	3.65±0.15	3.51 ±0.18	3.48±0.20
Tyrosine	1.28±0.07	1.25±0.11	1.65 ±0.05	1.31±0.03
Valine	2.51±0.14	2.44±0.13	2.40 ±0.09	2.48±0.12

[1] All results are expressed as averages of 5 rats ± SE of mean.
* Difference from group 1 ($P < 0.05$).

"warm" rats. None of the other liver components studied were affected by the amino acid imbalance. Furthermore, the activities of all the enzymes in the cold-exposed rats fed the basal diet were increased (group 2 vs. group 1). An increase of a similar magnitude of some of these enzymes was recently reported from this laboratory (2, 14).

The data in table 2 show the effect of the imbalance on concentrations of free amino acids in the plasma. The levels of alanine, arginine, aspartic and glutamic acid, lysine, methionine and phenylalanine were increased in the "warm" group fed the imbalanced diet (group 3 vs. group 1), and among these, lysine was the only indispensable amino acid, other than methionine and phenylalanine, that was increased. In contrast, no significant change was detected in the plasma amino acid levels in the comparable cold-exposed rats, despite the fact that the animals ate twice as much as their "warm" counterparts. Since the animals in the cold grew more rapidly, they apparently incorporated more of the ingested amino acids into proteins, thus maintaining the amino acid concentration in the plasma comparable to that observed in the controls. Furthermore, it may be also assumed that the cold-exposed animals oxidized the excessive amino acids and utilized them for heat production. This assumption was tested and confirmed in experiment 3, as shown by the data summarized in figures 2 – 4.

The cold-exposed animals fed the imbalanced diet incorporated radioactivity from methionine into CO_2 at a greater rate than did the corresponding "warm" animals, and at the end of the collection period the cold-exposed animals expired 72% more radioactivity than the "warm" ones (fig. 2). A more striking difference in the expired activity between these 2 groups was observed after administration of phenylalanine (fig. 3). In this case the cold-exposed animals expired almost 300 % more radioactivity than the corresponding group of "warm" animals. In contrast, there was no difference between the 2 groups in the radioactivity originating from the administered alanine (fig. 4). The "warm" animals fed the imbalanced diet also incorporated more radioactivity,

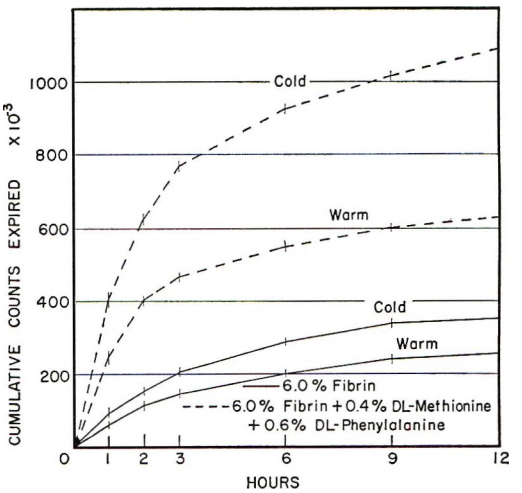

Fig. 2 Effect of amino acid imbalance and cold exposure on the cumulative recovery of 2 μc of intraperitoneally administered methionine-1-C^{14} as expired $C^{14}O_2$. Each point represents the average of 2 animals. Vertical lines represent the range. Percentage of the injected dose recovered as CO_2: "Warm"—control, 13.2, imbalanced, 31.7; cold-exposed—control, 18.1, imbalanced, 54.6.

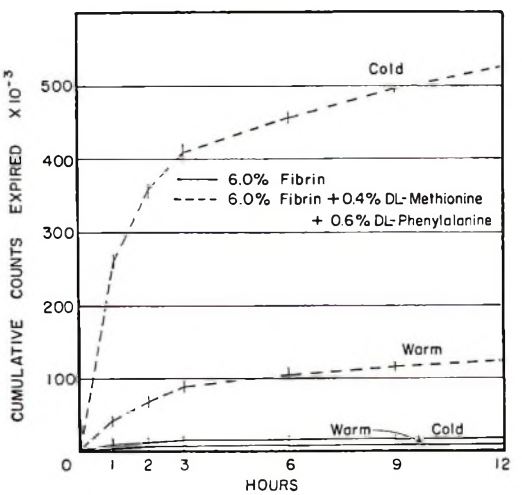

Fig. 3 Effect of amino acid imbalance and cold exposure on the cumulative recovery of 2 μc of intraperitoneally administered uniformly labeled phenylalanine-C^{14} as expired $C^{14}O_2$. Each point represents the average of 2 animals. Vertical lines represent the range. Percentage of the injected dose recovered as CO_2: "Warm"—control, 0.7, imbalanced, 6.7; cold-exposed—control, 1.5, imbalanced, 26.8.

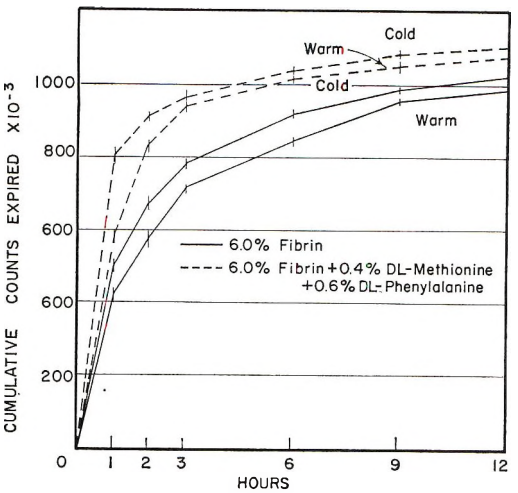

Fig. 4 Effect of amino acid imbalance and cold exposure on the cumulative recovery of 2 μc of intraperitoneally administered uniformly labeled alanine-C^{14} as expired $C^{14}O_2$. Each point represents the average of 2 animals. Vertical lines represent the range. Percentage of the injected dose recovered as CO_2: "Warm"—control, 42.4, imbalanced, 54.6; cold-exposed—control, 44.9, imbalanced, 55.1.

either from methionine or phenylalanine, into CO_2 than the animals fed the basal diet.

These data demonstrate that ingestion of the imbalanced diet leads to an increased catabolism of the imbalancing amino acids. The rat apparently uses this mechanism in an attempt to maintain a correct ratio in the amino acid pool for the protein synthesis. The rats kept in a warm environment may not be able to oxidize effectively the imbalancing amino acids, possibly because they are unable to dissipate all of the generated heat. However, when these rats are exposed to a low environmental temperature the catabolized portion of the amino acid mixture can be easily dissipated in the form of heat, and the remaining balanced portion can be effectively used for the formation of new tissues.

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Serum Lipid Levels, Fat, Nitrogen, and Mineral Metabolism of Young Men Associated with Kind of Dietary Carbohydrate[1]

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ABSTRACT Controlled diets providing daily approximately 2800 kcal, 37% of which were supplied by fat and 22% by either rice or sucrose, were fed to 6 healthy young men for 51 days. Serum cholesterol levels with the controlled diets were higher than the initial prestudy levels but there was no significant difference in serum cholesterol levels between the 2 controlled diets (rice vs. sucrose). The shift in source of 40% of the dietary carbohydrate from rice to sucrose did not significantly affect the levels of phospholipids and magnesium in the blood serum. No significant correlation was noted between blood glucose levels and the levels of each of the following blood serum constituents: cholesterol, phospholipids, and magnesium, nor between serum magnesium and either cholesterol or phospholipid levels. There was a highly significant correlation between serum cholesterol and phospholipid levels ($r = +0.940$). The substitution of sucrose for rice in the diets resulted in no significant differences in retention of nitrogen and phosphorus. The retention of calcium and magnesium was significantly lower when sucrose replaced rice. This difference could be attributed mainly to the higher content of both minerals in rice.

In recent years the influence of carbohydrate on blood lipid levels has come under scrutiny. Marshall et al. (1) observed that adult rats, fed a diet in which sucrose provided the carbohydrate, had higher serum cholesterol levels than rats receiving similar diets containing cornstarch. Kritchevsky et al. (2) reported that in chickens fed diets containing 54% of starch, glucose, or sucrose, highest serum cholesterol values were produced by the sucrose diets. Keys et al. (3) observed that diets, containing sucrose and milk carbohydrate to provide 17% of the calories, produced higher serum cholesterol levels in their schizophrenic subjects than did diets in which an equivalent amount of carbohydrate was provided by a mixture of fruit, vegetables, and legumes. From the results of further work in the same laboratory, Anderson et al.[3] concluded that the carbohydrate of legumes caused lower serum cholesterol than did sucrose. The substitution of the carbohydrate from a mixture of wheat flour and potatoes for sucrose had no significant effect on serum cholesterol levels.

In the study reported here, healthy young men were fed controlled diets made up of ordinary foods. The object of this study was to observe the effect on the levels of serum cholesterol, serum phospholipids, serum magnesium, and blood glucose, and on the metabolism of fat, nitrogen, and certain minerals when a major source of the carbohydrate in a nutritionally adequate diet was first white rice and then sucrose.

EXPERIMENTAL PROCEDURE

The subjects were 6 male student volunteers between 20 and 30 years of age, 170 and 186 cm in height, and between 67 and 85 kg in weight. They were examined and declared to be healthy by a physician.

The study, conducted from January to April at the University of Arkansas, was made up of a 5-day foreperiod and a 51-day controlled diet period. During the foreperiod the subjects ate self-selected

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[2] Present address: Human Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.
[3] Anderson, J. T., F. Grande and A. Keys 1960 The effect of carbohydrates of leguminous seeds, wheat, and potatoes on serum cholesterol in man. Federation Proc., *19:* 18 (abstract).

diets and recorded their total food intake in household measures. During the controlled diet period (10 consecutive 5-day collection periods) they consumed weighed diets in the laboratory.

The controlled diets were adaptations of a standardized diet, developed by Meyer et al. (4), adjusted to meet the nutrient needs of young men. These diets supplied approximately 2800 kcal and 8.5 g of nitrogen/day. The dietary fat (largely butterfat) provided 37% of the calories. Dietary carbohydrate provided 55% of the calories from the following sources: (in per cent) rice and sucrose, 25; wheat and oats, 18; fruit, 6.5; potatoes, beans, and celery, 5; and milk, 0.5.

During the first 25 days of the controlled diet period, fully milled long grain rice (not converted rice) and sucrose made up 40 and 5%, respectively, by weight of the dietary carbohydrate (providing 22 and 3%, respectively, of the calories). The mean daily intake of rice was 182 g (uncooked weight). During the last 26 days, 159 g of sucrose and 4.2 g of casein were substituted for 164 g of rice. The rice and sucrose then provided 5 and 40%, respectively, of the dietary carbohydrate (providing 3 and 22%, respectively, of the calories).

During the last half of the controlled diet period the caloric intakes of 3 subjects were increased by about 300 kcal/day to prevent weight loss. This increase was made by adding extra sucrose and fat in such quantities that the proportion of calories from fat remained constant throughout the study.

Samples of fasting venous blood were drawn from the subjects 27 days before they began the controlled diet period, on the first day of the controlled diet period, on the last day of the rice phase, and on the last day of the sugar phase. A portion of each sample was reserved for glucose analysis (5). The serum was analyzed for cholesterol (6), phospholipids (7), and magnesium (8).

Urine and feces were collected quantitatively throughout the controlled diet period. Daily urinary creatinine excretion was determined for each subject. Aliquots of five 24-hour urine collections were combined and a 5-day composite of feces was prepared for each subject, each period. A 5-day composite made up of weighed portions of the food served in the diets was prepared for the group of subjects each period. Food and feces were analyzed for fat; and food, feces, and urine were analyzed for total nitrogen, phosphorus, calcium, and magnesium. The fat, nitrogen, phosphorus, calcium, and magnesium content of the self-selected diets and the energy and fatty acid content of the self-selected and controlled diets were calculated. The analytical methods for fat, nitrogen, and minerals and the tables of food composition were those used in a previous study (9).

The first 5 days of each of the controlled diet periods were allowed for adjustment to the diet. Data from the following four 5-day periods of the regimen were used in calculating the mean fecal fat excretion and the mean nitrogen and mineral balances.

RESULTS AND DISCUSSION

With the controlled diets, the men had a lower mean intake of each nutrient studied, except magnesium, than they did with their self-selected diets. One of the greatest differences was in nitrogen intake which decreased from 18.4 g/day with the self-selected diets to 8.7 g/day with the controlled rice-containing diet. They also underwent a decrease in energy intake of about 500 kcal/day. Three men (subjects A, C, and E) were able to maintain their weight with this reduced intake. The other 3 showed small weight losses until their caloric intake was increased by about 300 kcal/day. The mean weight change for the entire dietary period was −0.25 kg.

Blood components. Mean values for serum cholesterol, serum phospholipids, serum magnesium, and blood glucose of the 6 men determined on blood samples drawn at the times previously designated are shown in table 1.

There was no significant difference between the mean serum cholesterol levels determined on the first and second (pre-controlled diet) samples (178 and 179 mg/100 ml). After consuming the rice diet for 24 days, the men had elevations in serum cholesterol ranging from 2 to 40

TABLE 1

Levels of blood components of six young men consuming self-selected and controlled diets

Blood component	Blood sample no.[1]	Diet	Subject						Mean [2]
			A	B	C	D	E	G	
			mg/100 ml						*mg/100 ml*
Serum cholesterol	1	Self-selected	140	174	116	188	218	232	178 ± 18
	2	Self-selected	142	164	116	176	222	252	179 ± 21
	3	Controlled, rice	168	178	156	206	222	246	196 ± 14
	4	Controlled, sugar	142	206	146	208	244	266	202 ± 20
Serum phospholipids	1	Self-selected	182	187	161	207	214	238	198 ± 11
	2	Self-selected	197	177	150	203	219	247	199 ± 14
	3	Controlled, rice	192	181	182	219	214	238	204 ± 9
	4	Controlled, sugar	163	224	169	221	241	251	212 ± 15
Serum magnesium	1	Self-selected	332	162	238	432	245	411	303 ± 43
	2	Self-selected	184	203	206	219	144	165	187 ± 12
	3	Controlled, rice	175	207	226	147	149	161	178 ± 13
	4	Controlled, sugar	160	115	188	148	134	183	155 ± 12
Blood glucose	1	Self-selected	102	88	88	80	108	95	94 ± 4
	2	Self-selected	88	88	80	86	86	88	86 ± 1
	3	Controlled, rice	92	83	83	74	92	83	84 ± 3
	4	Controlled, sugar	92	88	88	88	105	92	92 ± 3

[1] Blood samples were drawn on the following dates: no. 1, January 13; no. 2, February 9; no. 3, March 4; no. 4, March 30.
[2] Mean ± SE.

mg/100 ml giving a mean level of 196 mg/100 ml, an increase of 10% over their mean pre-controlled diet levels. At the end of the sugar diet period, subjects A and C showed decreases (26 and 10 mg/100 ml, respectively) but the other 4 subjects had further increases of from 2 to 28 mg/100 ml, resulting in a mean level of 202 mg/100 ml, an increase of 13% over the mean pre-controlled diet levels. The means of 196 and 202 mg/100 ml were not significantly different from each other. This might indicate that factors other than the shift in source of 40% of the dietary carbohydrate from rice to sucrose were influencing the serum cholesterol levels.

There were no significant differences among the mean serum phospholipid values found in any of the samplings. The serum phospholipid values were highly correlated with the serum cholesterol values ($r = +0.940$) but no significant relationship was found between serum phospholipids and any of the other blood constituents which were studied.

There was no significant difference among the mean serum magnesium levels of the last 3 samplings that were drawn at the beginning of the controlled diet period, at the end of the rice phase, and at the end of the sugar phase. The mean level of the first blood sampling, drawn 27 days before the controlled diet period, was significantly higher than the mean levels of the 3 subsequent samplings (303, as compared with 187, 178, and 155 mg/100 ml, respectively). The higher mean of the first sampling was caused by high serum magnesium levels of 3 subjects (332, 432, and 411 mg/100 ml). At subsequent samplings their serum magnesium levels had fallen to values which resembled more closely those of the other subjects. Serum magnesium levels did not correlate significantly with serum phospholipids or serum cholesterol.

The mean blood glucose level of the men after the sugar diet (fourth blood sampling) was not statistically different from the mean level after the rice diet (third blood sampling). The difference was similar in magnitude to the difference between the mean levels of the first 2 samplings drawn while the subjects were consuming self-selected diets. There was no significant correlation between blood glucose levels and any of the other blood constituents studied.

TABLE 2

Mean daily intake and excretion of fat, and intake and retention of nitrogen, phosphorus, calcium and magnesium by 6 men

Diet	Energy intake[1]	Nutrient									
		Fat		Nitrogen		Phosphorus		Calcium		Magnesium	
		Intake	Excretion	Intake	Retention	Intake	Retention	Intake	Retention	Intake	Retention
	kcal	*g*	*g*	*g*	*g*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Self-selected diets, 5 days[1]	3273	152.8		18.4		1711		1024		283	
Controlled diets											
Rice, 20 days (days 6–25 inclusive)	2788	115.2	2.7 ± 0.2[2]	8.7	0.6 ± 0.2[2]	1320	10 ± 24[2]	884	6 ± 23[2]	523	53 ± 12[2]
Sugar, 20 days (days 31–50 inclusive)	2985	121.5	3.2 ± 0.2[2]	8.2	0.9 ± 0.1[2]	1143	20 ± 25[2]	810	−56 ± 20[2]	387	20 ± 6[2]

[1] Values for nutrient content of the self-selected diets and for energy content of the self-selected and controlled diets were calculated.
[2] Mean ± SE (24 determinations).

Fecal fat excretion (table 2). The mean fat excretion was significantly higher ($P < 0.05$) with the sugar diet than with the rice diet. There was also a statistically significant difference ($P < 0.05$) between the apparent digestibility of the fat of the sugar diet (97.4%) and of the rice diet (97.7%) indicating that the higher fat intake with the sugar diet (when the caloric intakes of some subjects were increased) did not account entirely for the higher fat output with this diet. However, since the mean differences in fat excretion (0.5 g) and in fat digestibility (0.3%) between the 2 diet periods were so small, they are probably of little practical importance.

Nitrogen and mineral balance (table 2). There was no significant difference between either nitrogen or phosphorus retention with the rice and the sugar diets. With both nutrients, the subjects maintained positive balances close to equilibrium. The retention of both calcium and magnesium was significantly greater ($P < 0.05$) with the rice diet than with the sugar diet. Other investigators (10) have demonstrated with rats that calcium absorption was enhanced by a number of carbohydrates including lactose, cellobiose, and xylose, but it was not affected by glucose, galactose, fructose, or sucrose. Also in rats, lactose caused greater retention of magnesium than did starch or sucrose (11). In the present study no significant dietary effect on either urinary or fecal excretion of calcium or magnesium was demonstrated. Most of the difference in retention of each mineral could be traced to the higher content in the rice.

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Vitamin A in Serum and Liver of Weanling Pigs Fed a Protein-deficient Diet and Fixed Intakes per Unit of Live Weight of Carotene in Oil[1]

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ABSTRACT Pigs, 7 sets of 4 littermates, 10 to 13 weeks of age and partially depleted of their vitamin A stores, were each fed for 12 weeks equalized intakes of either 8 or 16% protein ration consisting mainly of soybean meal, cornstarch and oil, and vitamin and mineral fortified plus one of two daily intakes of β-carotene in oil, 88 or 440 μg/kg live weight. Pigs fed the 8% ration gained in live weight more slowly and required more feed per kilogram of live weight gain and had lower total hemoglobin and hematocrits than pigs fed the 16% ration. Although carotene fed with the 8% protein ration was essentially as effective in the maintenance of equivalent serum vitamin A concentrations as similar amounts fed with the 16% protein intake, in the case of either concentration or total amount of vitamin A in the liver, carotene fed with the low protein ration was more effective. Although the livers of pigs fed the high protein ration contained greater dry matter, lipids and nitrogen, expressing liver vitamin A concentration per unit of either lipids or nitrogen changed but little the above estimate for liver. The 8% protein ration resulted in lower serum total protein and albumin concentrations and higher serum α- and γ-globulin concentrations.

Despite an apparent adequacy of dietary carotene, low concentrations of vitamin A in the serum have been observed (1) in pigs fed low protein diets containing alfalfa meal as a source of carotene. Subsequent research with the dog [4] and sheep (2) has given essentially similar results.

The quantity and quality of protein fed with carotene also affects the storage of vitamin A in the liver. In the majority of research, as recently discussed by Berger et al. (3), this deposition has been observed to increase when the amount of dietary protein was increased, or when the quality has been improved either by a change of protein source or by adequate supplementation of a poor quality protein with the appropriate amino acid(s). No effects of protein-deficient diets on liver storage of vitamin A were observed in sheep (2) or in rats.[5] In possible opposition, relatively high intakes of protein have resulted in decreased deposition of vitamin A in the liver of chicks (4) and, possibly, similar effects in the case of rats (5).

Apparently, lowered concentrations of vitamin A in the serum of protein malnourished animals are a result of insufficient quantities of serum proteins which are necessary for the transport of vitamin A (6). In support of this, Friend et al. (1), Anderson et al. (2) and others,[6] have reported positive associations between serum albumin and vitamin A concentrations. In contrast, the cause of altered liver vitamin A deposition in protein malnutrition remains unexplained (5, 7).[6,7]

Because of the limited data with respect to deficient protein intake and serum vitamin A concentrations and the possibly conflicting reports on liver deposition of vitamin A in experimental animals fed protein-deficient diets supplemented with carotene, the present investigation was

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undertaken to evaluate quantitatively the effect of deficient protein intake on these 2 variables. Secondary to the above objectives was to determine the effects of deficient protein intake on serum protein fractions and the degree of correlation of these fractions with serum vitamin A concentration.

EXPERIMENTAL

Animals and feeding Weanling pigs (table 1) obtained from farms in Lancaster and Chester Counties, Pennsylvania, during the period January–March, 1962, were placed in individual wood shavings-bed concrete-floor pens providing floor space of 2.9 by 0.8 m. Each pig received a 16% protein ration (table 2) at an initial once daily rate of 3% of the average live weight of each set of 4 littermates. This amount was increased by 0.14 kg at successive 7-day periods. Water was provided 3 times daily and during the initial 2 to 3 weeks, small and decreasing quantities (0.5 to 0.1 kg) of reconstituted skim milk were mixed with the 16% protein ration allowance. Pigs were vaccinated for hog cholera and were wormed during the second to fourth weeks.

During the above partial vitamin A depletion period, blood was obtained from the anterior vena cava of each littermate at successive 14-day periods. Two weeks after the average serum vitamin A concentration for each litter had decreased to or less than 20 μg/100 ml, each littermate received, according to a previously randomized allotment, one of 4 diets arranged according to a 2 × 2 factorial design. These were either a deficient, 8%, or an adequate, 16%, protein ration (table 2) and one of two daily carotene intakes per kilogram of live weight, 88 or 440 μg of β-carotene in a specially processed fraction of a vegetable oil.[8] The adequate protein intake was based on an estimated average weight of the pigs during carotene-protein feeding of approximately 45 kg and was 3% greater than the requirements for finishing meat-type pigs as set forth by the National Research Council (8). The lowest carotene intake, 88 μg/kg of live weight, was that of the National Research Council for the above estimated live weight. The highest carotene intake was chosen such that the responses of plasma and log liver vitamin A concentrations on log carotene intake would be linear (9) and, thus, allow the use of parallel line bioassay procedures (10, 11) to quantitatively estimate the effects of protein intake on these variables. The ration allowance

[8] Vegetable Oil 1400, E. F. Drew Company, derived from coconut oil, containing no added antioxidants and having a maximal iodine number of 10. Every 1 to 2 weeks of the carotene-protein feeding period, the desired quantities of all *trans* β-carotene were dissolved in the oil (contained in brown glass jars) by mild heating and stirring under an atmosphere of nitrogen, and were stored at ca. 1°C. The amount required for each day's feeding was removed and allowed to come to room temperature prior to measurement and incorporation into the rations. These carotene-in-oil solutions were prepared by the Food Agricultural Products Division of Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. R. H. Bunnell.

TABLE 1

Pig performance during partial vitamin A depletion

Litter	Breed [1]	Sex [2]	Initial age	Live weight		Serum vitamin A		Duration of partial vitamin A depletion
		M – F		Initial	Daily change	Initial	Daily change	
		no.	*wk*	*kg*	*kg*	*μg/100 ml*	*μg/100 ml*	*weeks*
1	CB	4 – 0	4	5.0	0.36	21.5	−0.4	6
2	CB	0 – 4	5	6.3	0.36	34.0	−0.7	6
3 [3]	CB	2 – 1	6	7.9	0.33	23.6	−0.4	4
4	CB	0 – 4	7	9.1	0.36	28.3	−0.5	4
5	CB	2 – 2	8	12.0	0.40	29.9	−0.5	4
6	Y	4 – 0	5	7.0	0.35	37.0	−0.6	8
7	Y	0 – 4	5	6.4	0.32	36.2	−0.6	7
		Mean	5.7	7.6 ± 0.5 [4]	0.351 ± 0.002	30.3 ± 0.6	−0.55 ± 0.02	5.6

[1] CB = Crossbreeds predominately Yorkshire and Berkshire, Y = Yorkshire.
[2] M = male, F = female.
[3] A male pig in this litter died in the 4th week of partial vitamin A depletion. The data were not included in the statistics for this litter.
[4] SE.

TABLE 2

Composition of rations [1]

	8% Protein	16% Protein
	kg	*kg*
Soybean oil meal, 50% protein	14.20	29.70
Cornstarch	74.55	59.10
Corn oil	2.00	2.00
Dried beet pulp	2.00	2.00
Trace mineral mixture [2]	2.00	2.00
Dicalcium phosphate	2.40	2.00
Calcium carbonate	0.10	0.20
Iodized salt	0.50	0.50
DL-Methionine	0.25	0.50
Vitamin mixture [3]	2.00	2.00
Total	100.00	100.00

[1] Based on 10 samples taken during the course of the experiment, the average percentage proximate composition of the 8% protein ration as fed, with SE of the means in parentheses, was: dry matter 11.46(0.51), crude protein 8.38(0.58), ether extract 2.11(0.12), crude fiber 1.02(0.06), nitrogen-free extract 73.60 (0.83) and ash 3.44(0.14), with a carotene content of 35 μg/kg (11). Similar statistics for the 16% protein ration were: 11.11(0.55), 15.94(0.69), 2.39 (0.08), 1.48(0.11), 64.89(0.90) and 4.19(0.09), with a carotene content of 108 μg/kg (22).

[2] Contributed the following mineral elements from feed grade sources per kg of ration: (in grams) Fe, 0.144; Zn, 0.023; Cu, 0.007; Mn, 0.016; and Co, 0.003 in a cornstarch premix.

[3] Contributed the following vitamins: (in mg/kg of ration) thiamine (M.N.,USP), 1.54; riboflavin (USP), 3.30; niacin (USP), 22; Ca-DL-pantothenate (USP), 26.4; pyridoxine·HCl (USP), 1.32; choline chloride (70%), 1,254; cyanocobalamin (1% in calcium phosphate), 0.022; *d*-α-tocopheryl acetate, 6.6; menadione sodium bisulfite, 0.55; and chlortetracycline (97.9%), 55; and vitamin D_2 in a cornstarch premix, 440 IU.

during this period of carotene-protein feeding was continued as during partial vitamin A depletion, but increased at successive 7-day periods by only 0.09 kg. This reduction was to preclude possible feed refusals by pigs fed the 8% protein ration.

The amount of carotene in oil given to each pig was based on its anticipated average live weight for the particular 7-day period, the latter calculated from the previous two 7-day live weights. To insure complete consumption of the daily allowance of carotene in oil, this was thoroughly mixed with a small portion (0.473 to 0.946 liter) of each pig's previously weighed 8 or 16% protein ration allowance and fed first. After consumption of this, the remainder of the ration was given.

The carotene-protein feeding for each set of 4 littermates was 12 consecutive 7-day periods, after which each pig was slaughtered.

Observations and analyses. With the exception of carotene in oil which was measured to the nearest 0.5 ml by syringe, all feeds fed or refused were weighed daily to the nearest 45 g, but reported in kilograms. No feed refusals occurred during carotene-protein feeding. Samples of the 2 protein rations were obtained at approximately 4-week periods for proximate analyses as well as carotene content by AOAC procedures (12), the results reported in footnote 1 of table 2. Prior to feeding each batch of oil, the carotene content of a petroleum ether (30 to 60°C) solution of the oil was determined in a photoelectric colorimeter,[9] using a 440-mμ filter and all *trans* β-carotene[10] as a reference standard. The average carotene concentration of the oils, based upon analyses of 13 batches for each concentration, were for the 88-μg carotene intake group 110 ± 3 μg/g and for the 440-μg carotene intake group 476 ± 9 μg/g.

Daily average minimal and maximal barn temperatures were, respectively, 12° and 19°C with ranges between −7 and 26°C and zero and 31°C.

Venous blood samples were obtained by puncture of the anterior vena cava every 14 days during partial vitamin A depletion and every 28 days thereafter. The blood was allowed to clot at barn temperature for approximately 30 to 60 minutes, held at ca. 1°C for another 60 minutes and then centrifuged to obtain the serum. Total hemoglobin (13) and hematocrit (14) were determined terminally on venous blood using ethylenediaminetetraacetic acid, disodium salt, as an anticoagulant. Serum vitamin A concentration was determined by the Kimble procedure (15) except that the alcohol used to precipitate the proteins was added drop by drop with vigorous agitation (1). Serum protein fractions were separated by a standard electrophoresis technic.[11] The various zones were cut out and eluted in a measured volume of 0.5% aqueous sodium carbonate by gentle agitation for 10 minutes, followed by a 60-minute period to allow the fibers to settle. The relative percentages were then calculated from optical densities obtained by reading in a photoelectric colorimeter [9]

[9] Evelyn photoelectric colorimeter, series 4600.

[10] Hoffmann-La Roche, Inc., Nutley, New Jersey, and supplied through the generosity of Dr. R. H. Bunnell.

[11] A method for serum protein using bromphenol blue dye in alcoholic solution (Spinco "Procedure B"). Tech. Bull. no. TB 6050 A. Spinco Division, Beckman Instruments, Inc., Palo Alto, California, 1958.

with a 580-mμ filter, using as standards known concentrations of bromphenol blue in 0.5% aqueous sodium carbonate. Total protein in the serum was determined by the biuret reaction (16). From this and the relative percentages, the concentration of each fraction was calculated.

At slaughter, the liver and both kidneys were removed, weighed, homogenized in a Waring Blendor and sampled, with the samples held at ca. −18°C for subsequent analyses. A modified Gallup-Hoefer procedure (17) was used to determine the carotenoids and vitamin A content of the liver and the procedure of Gade and Kadlec (18) for the kidney. Dry matter and nitrogen of liver were determined by AOAC procedures (12). Lipids of liver were determined by extracting for 20 hours with hot ethanol, diluting the alcohol extract with an equal volume of water, and extracting the resulting solution with hexane. An aliquot of the hexane extract was evaporated in previously tared aluminum cups under nitrogen on a steam bath; the cups were placed in a vacuum oven for one hour at 50°C, and then weighed.

Data were subjected to analysis of variance for the purpose of isolating variation among replicates (sets of littermates), among treatments and remainder (error). Prior to the beginning of the experiment, the among treatments variation was further divided into 2 sets of contrasts. The first included variation (a) due to 8 vs. 16% protein intakes; (b) 88 vs. 440 μg carotene intakes; and (c) the interaction of these. The second, intended only for serum and log liver vitamin A concentrations, followed that as outlined by Bliss (10) for a two-dose factorial parallel line bioassay and was for the purpose of estimating the effects of 8 vs. 16% protein intakes on these response criteria.

One male pig in litter 3 died due to hog cholera[12] the week prior to carotene-protein feeding. Accordingly, the values for this pig were estimated by the procedures cited by Bliss (10) and, with the exception of the actual liver vitamin A concentrations, were included in the computation of the reported statistics.

RESULTS

Although all pigs within sets of 4 littermates received and consumed the same daily feed intake (table 3), those that were fed the 8% protein ration gained in live weight at a slower average daily rate ($P < 0.001$) and required greater amounts of feed per unit gain in live weight ($P < 0.001$) than pigs fed the 16% protein ration. Total hemoglobin and hematocrit values were also lower during the terminal or twelfth week of carotene-protein feeding for those pigs fed the 8% protein ration ($P < 0.05$ and $P < 0.01$).

[12] We are indebted to Dr. Klaus Hubben, School of Veterinary Medicine, University of Pennsylvania, for this diagnosis.

TABLE 3

Effect of percentage of protein in the ration and carotene intake upon live weight gains, feed conversion, hemoglobin and hematocrit of pigs

	8% Protein		16% Protein		SD/ animal
	88 μg of carotene [1]	440 μg of carotene	88 μg of carotene	440 μg of carotene	
Live weight, kg					
Initial	21.9	21.7	21.7	21.6	1.0
Average	41.2	41.0	47.3	46.6	1.6
Daily gain	0.51	0.51	0.65	0.64	0.03
Feed intake/day, kg	1.66	1.66	1.66	1.66	— [2]
Feed required/unit gain in live weight, kg/kg	3.25	3.26	2.57	2.61	0.17
Terminal total hemoglobin, g/100 ml	13.8	13.7	15.0	14.7	1.2
Terminal hematocrit, % red blood cells	44.0	45.3	49.3	49.9	3.4

[1] Daily intake/kg live weight
[2] Range for 7 litters between 1.45 and 2.00 kg/day.

Serum vitamin A concentrations (table 4) tended to be maintained during the course of carotene-protein feeding (average values) and at the termination of this period (terminal values) at essentially the same average level in pigs fed the 16% protein (average of both carotene intake groups) as those fed the 8% protein. Using parallel line bioassay procedures, in which the linear responses of average serum vitamin A values on the log of carotene intakes were derived, it was found that the carotene fed with the 8% protein ration was 0.8 times as active in maintaining identical serum vitamin A levels as equivalent amounts of carotene fed with the 16% protein ration. Confidence limits (95%) for this ratio were between 0.5 and 1.2. A similar ratio for the terminal serum values was 0.8 with limits between 0.4 and 1.2. There appeared to be an interaction between percentage protein in the ration and carotene intake as evidenced by the absence of a difference in serum

TABLE 4

Effect of percentage of protein in the ration and carotene intake upon vitamin A in the serum, liver and kidney of pigs

	8% Protein		16% Protein		SD/ animal
	88 μg of carotene [1]	440 μg of carotene	88 μg of carotene	440 μg of carotene	
Serum vitamin A, μg/100 ml					
Initial	9.9	10.6	9.4	10.4	2.8
Average	18.8	28.5	18.4	31.8	3.6
Terminal	20.9	30.3	20.7	34.4	4.4
Terminal liver					
Weight, g	832	886	1186	1156	90
Weight/unit live weight, g/kg	12.5	13.5	15.2	15.1	1.4
Carotenoids					
Conc, μg/100 g					
Actual	12	26	6	15	—
Log	1.04	1.39	0.75	1.05	0.18
Vitamin A					
Conc, μg/100 g					
Actual	1019	5137	341	3852	—
Log	2.92	3.69	2.44	3.55	0.22
Total, μg					
Actual	8111	44369	4057	44567	—
Log	3.83	4.64	3.51	4.61	0.21
Per unit live wt, μg/kg					
Actual	122	672	52	564	—
Log	2.01	2.82	1.62	2.73	0.21
Terminal kidney					
Weight, g	164	167	220	228	12
Weight/unit live wt, g/kg	2.46	2.52	2.83	2.96	0.13
Carotenoids, μg/100 g	9	9	8	8	4
Vitamin A					
Conc, μg/100 g	32	51	26	57	12
Total, μg	52	84	58	127	18
Total liver plus kidney vitamin A, μg					
Actual	8163	44453	4115	44696	—
Log	3.84	4.64	3.52	4.61	0.21

[1] Daily intake/kg live weight.

vitamin A levels between pigs fed the deficient and adequate protein rations at the lowest carotene intake, 88 μg, and by the presence of a difference at the highest carotene intake, 440 μg. However, statistical evidence was lacking, since $P > 0.05$ for the divergence in slopes between the 2 protein intake groups.

Liver vitamin A values expressed as concentration per unit weight of liver, or total amount, or concentration per unit of live weight are presented in table 4. The pigs fed the 8% protein ration had higher average values (across both carotene intake groups) than those pigs fed the 16% protein ration. Based upon parallel line bioassay procedures, in which log concentration of vitamin A/100 g of liver was used as the criterion of response, carotene fed with the 8% protein ration was 1.7 times as effective in the maintenance of similar liver vitamin A stores as equivalent amounts of carotene fed with the 16% protein ration, with 95% confidence limits between 1.3 and 2.4. Similar values based upon log total amount and log concentration per kilogram of live weight were, respectively, 1.3 and 1.5 with confidence limits between 1.0 and 1.8 and between 1.1 and 2.0. In contrast with serum vitamin A, the differences in response of liver vitamin A to protein intake were greater at the lower carotene intake, 88 μg than at the higher intake, 440 μg. Although this inconsistency was not statistically significant (since $P > 0.05$ for the divergence in slopes between protein intakes), in the case of log concentration of vitamin A/100 g of liver, it approached significance at $P < 0.05$.

The vitamin A concentration per unit weight of the kidney (table 4) differed inappreciably between protein intakes. Because of the greater weights of the kidneys in those pigs fed the 16% protein ration, the total amount of vitamin A in this organ was therefore greater ($P < 0.01$) than in those of the pigs fed 8% protein. The total amount of vitamin A in both the liver and kidneys (last 2 rows of table 4) as contrasted with the total amount of the vitamin in the liver (rows 10 and 11, table 4) was increased only slightly, indicating the small contribution of the kidney to the total stores of vitamin A in these 2 organs.

To possibly relate differences observed in vitamin A concentration of the livers to dry matter, lipid or nitrogen content of the liver, these constituents were determined and presented in table 5. Pigs fed the high protein intake had livers containing greater concentrations of dry matter ($P < 0.001$), lipids ($P < 0.05$) and nitrogen ($P < 0.01$) (table 5). Similar results were also apparent for total amounts of these 3 constituents in the liver ($P < 0.001$). When the lipid content of the

TABLE 5

Effect of percentage of protein in the ration and carotene intake upon dry matter, lipids and nitrogen in livers of pigs

	8% Protein		16% Protein		SD/ animal
	88 μg of carotene [1]	440 μg of carotene	88 μg of carotene	440 μg of carotene	
Dry matter					
Concentration, g/100 g	27.2	27.4	28.5	28.4	0.6
Total, g	227	243	338	328	28
Lipids					
Concentration, g/100 g	3.90	3.71	4.00	4.09	0.29
Total, g	32	32	47	47	2
Concentration/unit dry matter, g/100 g dry matter	14.4	13.5	14.0	14.4	1.1
Nitrogen					
Concentration, g/100 g	3.10	3.01	3.31	3.39	0.20
Total, g	26	26	39	40	2
Concentration/unit dry matter, g/100 g dry matter	11.4	11.0	11.6	11.9	0.7

[1] Daily intake/kg live weight.

livers was expressed as concentration per 100 g of dry matter, there were no statistically significant differences among dietary groups. When nitrogen was expressed in the same manner, there was still a difference ($P < 0.05$) in favor of the pigs fed the 16% protein ration. However, when vitamin A content in μg/100 g of each of the 3 constituents was calculated and subjected to parallel line bioassay procedures in which log liver vitamin A concentration per 100 g of liver lipids on log carotene intake and log vitamin A concentration per 100 g liver nitrogen on log carotene intake were used, the ratios obtained were only slightly higher (0.1) than the ratio calculated on the basis of log liver vitamin A concentration per 100 g of liver. It therefore appears that neither liver lipids nor liver nitrogen content can explain much of the difference in liver vitamin A deposition between pigs fed the adequate and deficient protein rations. The ratio based upon vitamin A concentration in the liver per unit dry matter was not calculated, since the divergence in slope was significant ($P < 0.05$), thus invalidating the assay.

Serum protein concentrations (table 6) were affected by the percentage of protein in the ration.[13] Total serum protein was less ($P < 0.001$) in those pigs fed the 8% protein ration which was also true for the albumin concentration ($P < 0.001$). In contrast, α-globulin was greater ($P < 0.05$) as well as γ-globulin (average values, $P < 0.01$), with the average β-globulin fraction concentrations remaining essentially the same between the 8 and 16% protein ration groups. The above mentioned differences were reflected in the smaller ($P < 0.001$) albumin-to-globulin ratios in the pigs fed the 8% protein ration.

With one exception, the simple correlations between serum vitamin A and any

[13] Unless otherwise stated, the probabilities (P values) in this paragraph refer to both the average and terminal values observed during carotene-protein feeding.

TABLE 6

Effect of percentage of protein in the ration and carotene intake upon the concentration of serum proteins of pigs

	8% Protein		16% Protein		SD/ animal
	88 μg of carotene [1]	440 μg of carotene	88 μg of carotene	440 μg of carotene	
Total protein, g/100 ml					
Initial	5.80	5.99	5.99	5.92	0.36
Average	5.56	5.56	6.48	6.49	0.22
Terminal	5.70	5.60	6.74	6.72	0.26
Albumin, g/100 ml					
Initial	3.40	3.38	3.64	3.51	0.36
Average	2.69	2.65	3.76	3.88	0.15
Terminal	2.71	2.61	3.87	3.98	0.25
α-Globulin, g/100 ml					
Initial	1.13	1.26	1.10	1.20	0.19
Average	1.26	1.30	1.18	1.17	0.09
Terminal	1.32	1.38	1.24	1.23	0.12
β-Globulin, g/100 ml					
Initial	0.74	0.81	0.78	0.76	0.06
Average	0.81	0.84	0.89	0.83	0.07
Terminal	0.82	0.85	0.91	0.82	0.10
γ-Globulin, g/100 ml					
Initial	0.53	0.54	0.47	0.45	0.09
Average	0.80	0.77	0.65	0.61	0.11
Terminal	0.85	0.76	0.72	0.69	0.14
Albumin-to-globulin ratio					
Initial	1.42	1.34	1.56	1.47	0.19
Average	0.94	0.93	1.40	1.52	0.11
Terminal	0.91	0.88	1.37	1.48	0.18

[1] Daily intake/kg live weight.

one of the serum protein fraction concentrations and the serum albumin-to-serum globulin ratio (for pigs fed the 88 μg or 440 μg carotene intakes or for all dietary groups) were of small magnitude and not statistically significant. The exception was a correlation of +0.62 between serum vitamin A and β-globulin within the highest carotene intake grouping.

DISCUSSION

Since serum levels of vitamin A were essentially the same in pigs fed the low protein ration and in pigs fed adequate protein, it is not apparent from this study that a low percentage of dietary protein affects serum vitamin A concentration. However, pigs fed the deficient protein diet, 8%, had greater concentrations and total amounts of vitamin A in the liver than those fed adequate protein, 16%, and thus had greater stores of the vitamin to maintain it in the serum. Inasmuch as when fixed carotene intakes are fed to growing animals, serum and log liver vitamin A concentrations are apparently in equilibrium as evidenced by a linear association between these 2 variables (19), it was possible to obtain an approximation of the magnitude of the differences in serum vitamin A levels at equal concentrations of liver vitamin A by solving linear regressions of the concentration of serum vitamin A in μg/100 ml on the log of liver vitamin A concentration in μg/100 g. These slopes, with standard errors, were for the 8% protein ration group, 11.3 ± 2.5, and for the 16% group, 10.6 ± 2.1, and simple correlation coefficients, respectively, of 0.79 and 0.83. These did not differ significantly, and therefore, a combined slope was computed which was 10.9 ± 1.6, with the simple correlation being 0.82. Under the assumption that similar linear slopes of serum vitamin A concentration on log liver vitamin A concentration existed for both protein groups as plotted in figure 1, it was found (from the vertical distance between the 2 parallel lines) that the serum vitamin A concentration of pigs fed the 8% protein ration was 5.4 μg/100 ml less than that of the pigs fed the 16% ration. Similar values, when liver vitamin A stores were expressed as log total micrograms and as log micrograms in the liver per kilogram of live weight, were 4.0 and 4.8, respectively. Although it is recognized that the estimates are only approximations because of the nature of the data (20), the reduction in serum vitamin A concentration occurring in protein deficiency is of sufficient magnitude to warrant additional study. Although severe protein deficiency results in a delay in the appearance of xerophthalmia (21), it would be pertinent to determine its effect upon changes characteristic of early hypovitaminosis A as cited by Nelson et al. (22), which are due presumably to insufficient vitamin A circulating in the serum.

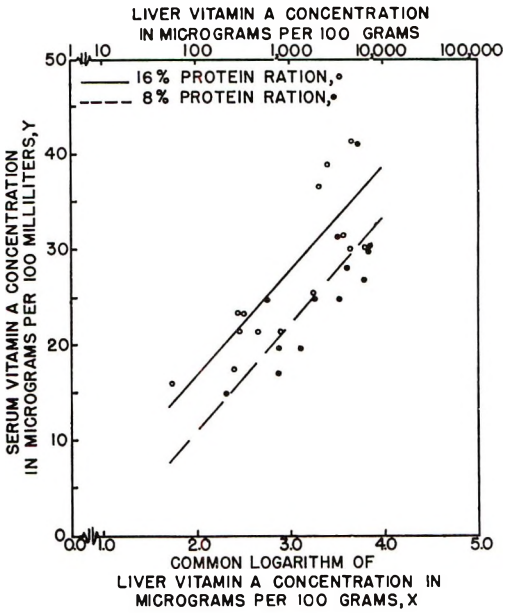

Fig. 1 Association between serum and log liver vitamin A concentrations in pigs fed 8 and 16% protein rations.

The difference in liver vitamin A storage in favor of the pigs fed the deficient, 8%, protein ration was unexpected in light of the observations by most researchers, as reviewed by Berger et al. (3), that a protein-deficient ration fed with carotene decreases liver deposition of vitamin A. Had it been possible to correct liver vitamin A stores for differences in growth as was done by Berger et al. (3) who utilized the rat in protein-carotene interrelationship studies, the differences between the values of the pigs fed the 8 and 16% protein

rations might have been changed to one in favor of the 16% group. However, this correction may be subject to question, since in chicks in which the actual live weights did not differ appreciably, liver vitamin A storage was found to be inversely related to protein intakes of 21.9 to 71.5% (23). In comparison of the data obtained in the present experiment with those of Friend et al. (1) and others (24), it is apparent that pigs fed the 8% protein ration were only mildly protein-deficient.

The direction of the effects of the protein-deficient diet on the distribution and concentration of the serum protein fractions was expected in light of previously reported investigations (1, 24). However, these responses, particularly the total protein concentration and the shift in the distribution and concentration of the fractions from albumin to globulins, although statistically significant at a high level of probability, were relatively small in magnitude and, thus, indicated the presence of only a mild protein deficient state of the pigs fed the 8% protein ration. In light of this, it was not unexpected that the degree of simple linear correlation between the concentrations of various serum protein fractions and serum vitamin A was not statistically significant. Since the decrease in albumin accompanied by an increase in globulins is not unique to protein deficiency but has been observed in vitamin A and E deficiencies (25, 26) and may be under endocrine control as reported by Beardwood et al. (27) as a response to stress, it appears that the level of vitamin A in the serum may be influenced by malnutrition caused by one or more deficiencies. This is in need of more critical investigation. It is recognized that the study reported herein dealt only with total vitamin A content of the serum and not with the alcohol and ester forms which are associated with different protein and lipoprotein fractions of the serum (6).

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Nutrient-to-Calorie Ratios in Applied Nutrition

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ABSTRACT This paper discusses the desirability of expressing the nutrient requirements of man and animals relative to their associated energy intake, and proposes a format for expressing a dietary standard for humans. Caloric needs are known to vary with size, activity, and productive performance. There is agreement that most of the nutrients quantitatively considered in diet or ration formulation should remain in balance with each other and with energy, for optimal food efficiency. Many nutritionists believe that it is not possible to state the requirements for individual nutrients without also specifying the amounts of others also to be ingested. In the case of nutrients that must be stored in the body, as during growth, the ratio to energy intake changes progressively to the final adult equilibrium status. In the case of the human, one adult, and 3 juvenile "diet" categories appear to be adequate, within each of which the interbalances between nutrients and that between nutrients and energy are constant. Within these 4 categories, adequate daily nutrition can be accomplished by adjustments in the intake of the diet as a whole.

One of the far-reaching concepts in dietetics and animal rationing is the importance of balance between the nutrients of the diet — the realization that relative excesses are no less undesirable than relative deficiencies. We have only begun to unravel the picture of nutrient interrelationships, but it is abundantly clear that the efficiency of food utilization by the animal body may be markedly affected by alterations in the inter-nutrient balance, or between the nutrient and energy pattern, or both. There may be some doubt whether we yet can define the ideal interrelations, but periodically since 1810 tabulations have been made of the quantities of energy and of nutrients that evidence indicated were needed daily by farm animals according to species, weight, age, sex and production performance.

One pertinent study was that by Guilbert and Loosli (1) in which they computed for farm animals and poultry from the appropriate feeding standards (NAS-NRC series) the recommended daily feed intakes, and total digestible nutrients (TDN); and per unit TDN, the digestible crude protein, calcium, phosphorus, carotene, vitamin A, vitamin D, thiamine, riboflavin, niacin and pantothenic acid for individuals at attained weights of from 10 to 100% of expected adult size. Although their study was mostly concerned with immature growing animals, and was hampered by excessive variability partly from the fact that the NAS-NRC feeding standard values at that time contained margins of safety of unknown magnitude over the true requirements, they prepared a table of generalized recommended allowances of protein, Ca, P, thiamine and riboflavin in ratio to TDN intake for farm livestock and poultry. They state, "The feasibility of expressing (in feeding or dietary standards — E.W.C.) the relationship of the various nutrients with metabolizable energy rather than with total digestible nutrients should be explored." They also comment, "In the preoccupation of seeking new nutritional factors, it would appear desirable to pause when they are found to determine their fundamental relations to other nutrients, to body weight, or to caloric intakes, and the quantitative requirements in a systematic procedure."

Because of incomplete knowledge, the authors of the early tabulations often claimed as requirements, nutrient values later shown to be excessive; and it is significant that, other than inclusion of additional nutrients, the chief changes in the chronologically successive "standards," even though by different authors, have been downward revisions of the amounts of nutrients believed to be required relative to energy intake. The criteria for such changes have been: increased efficiency of the ration in producing growth, fattening, milk or egg production, or de-

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creased mortality, or both, or evidence of dietary-induced morbidity — all of which have a direct bearing on the economics of the livestock enterprise.

In standards applicable to captive animals, the relationship that has been least modified with the passing of time has been that between energy and protein, although it was the first to appear. Indeed, it was the proportion of protein to "carbohydrate equivalent" that was the basis of Henry's (2) "nutritive ratio," a term coined by him in 1904. The term, "balanced ration," originally referred quantitatively only to its protein-to-energy ratio, although it was defined by Henry as: ". . . a combination of farm foods containing various nutrients in such proportion and amount as will nurture the animal for 24 hours with the least waste of nutrients."

Distortion of the protein-to-calorie ratio was more promptly reflected in animal performance than most other formula modifications, and quite naturally led to ascribing a greater importance to protein levels than to that of other nutrients. Because high protein feeds were more costly than those used primarily for energy, *minimal* protein levels compatible with maximal ration efficiency were understandably considered *optimal* levels.

Until the early 1950's, the practical expression of protein-energy balance in animal and poultry diets was the percentage of protein per unit weight of ration dry matter. The energy concentration of such rations for a given species deviated only between relatively narrow limits. Adjustments of energy intake according to desired rates of growth, fattening or production, or to meet the maintenance needs of animals of varying sizes, were made by regulating ration allowances. Since other nutrients than protein were contained in the ration mixture, they, too, were automatically consumed in fixed, although often unknown, ratios to calories, and to each other.

Increasing energy intake to obtain greater performance by allowing greater daily consumption of the diet mixture obviously has strict limitations, and about 1954, as an alternate method, poultry nutritionists began to increase the energy concentration of the diet by direct additions of edible fat. In the 1960 revision of the NRC publication on the requirements of poultry, the NRC Poultry Feeding Standard Committee (3) states, "It has been customary for research workers in poultry nutrition to express nutrient requirements in terms of nutrient per unit weight of ration .... This convenient method is inaccurate . . . (because) . . . protein requirement can be defined accurately only in relation to energy concentration . . . ."

The need for maintaining specified protein-to-calorie ratios in diets is not restricted to poultry rations. Stare (4), in summarizing the findings of a symposium on protein nutrition in 1958, comments: "The last point, and probably the most important, is the concept of the balanced diet or balanced nutrition . . ." ". . . the type of nutrition that supplies a sufficiency, but not an excess, of calories coming from both carbohydrates and fat . . . in adequate ratio with . . . vitamins, minerals and amino acids." During the same symposium, Johnston (5) stated: "The ratio of calories to protein is one of the most important problems . . ." and we cannot speak about the protein needs without taking calories into consideration. It is better not to speak of the amount of protein per kilogram daily, but to express it as the percentage of caloric intake." Hegsted (6), in his 1958 paper, "Protein Requirements in Man," states: "I . . . emphasize the rather high correlations between protein and caloric intakes. These are expected, but often ignored."

There is sound biological basis for considering caloric intake as the fundamental basis for the requirement of most of the known essential nutrients. It is concisely stated by Kleiber (7) in his review of dietary deficiencies and energy metabolism. He writes, "Since any dietary deficiency, in contrast to lack of food, means an imbalance of the ration, one may derive from Mitchell's hypothesis, a bioenergetic criterion for a dietary deficiency leading to the following definition: A diet is deficient in any nutrient whose addition decreases the calorigenic effect of the ration. One may expand this definition, and state that a ration is deficient (and hence unbalanced) in any food constituent whose addition increases the total efficiency of energy

utilization." This merely says that, the specific dynamic action of food represents waste energy, which can be minimized by proper balance of the energy and nutrient content of the diet.

In Kleiber's review (7), he finds evidence that changes in proportions to energy intake, of 1) potassium, magnesium, calcium, phosphorus, iron, iodine; 2) of ascorbic acid, thiamine, riboflavin, niacin (B vitamins as a group), probably of vitamins A, D, and E; and 3) of protein, affect metabolic rate. He partially summarizes these observations in his recent book (8) as follows: "Not only the requirements of food energy, but also that of protein and of most vitamins may be expressed per unit of the three-quarter power of body weight ($W_{kg}^{0.75}$), because these dietary requirements are directly related to energy metabolism." Brody (9) states: "As regards the maintenance needs for vitamins and trace elements, those that are involved in the general oxidation-reduction processes of intermediary metabolism, . . . undoubtedly vary directly with energy metabolism — that is, with the food energy consumption."

Adult protein-calorie ratios for maintenance living

Obviously, then, nutrient requirements may well be expressed relative to calorie needs, and it may be of interest at this point to compare the published maintenance digestible energy requirements of adults of different species with their digestible protein needs — the relationship which has been more widely and accurately established in biological research and in practical feeding than any other.

The conformity of the protein-to-calorie ratio for mammalian species, differing as widely in size as rats and cows, strongly supports the premise that digestible protein is required for adult animals at maintenance living in about the same ratio to digestible energy needs, regardless of species or size.

The common belief that sex, per se, affects the adult maintenance dietary requirement is probably unwarranted. It has been shown (10, 11), that for energy requirements, sex difference is primarily a size difference. Nor is any distinction made between sexes, per se, in ration formulation for the feeding of idle adult farm or laboratory animals.

Protein-to-calorie requirements of adults — super-maintenance living

Within the adult group within each species, there are subgroups that, because of their differing functions or activities, might conveivably require rations differing from those of simple maintenance living.

Pregnancy. During pregnancy there is a gradual increase in basal metabolism to a maximum, at term, of 120% of the nonpregnant state. There is also an accumulation of products of conception. Most of these changes are insignificant, nutritionally, until the last third of pregnancy. During the last trimester the voluntary activity of the mother is usually progressively reduced so that there may be only a relatively small, if any, increase in caloric needs for "work." In any case the maximal extra daily metabolizable energy intake needed by pregnant women probably does not exceed 520 kcal (11, p. 388).

As to protein, Terroine (12) estimates that in human pregnancy not more than one gram per day of nitrogen is required for the formation of the fetus, its adnexa, and the expected increase in body protein

TABLE 1

Grams digestible protein required daily per 1000 digestible kcal of dietary energy for adult maintenance

	Man [1]	Cow [2]	Horse [2]	Pig [3]	Sheep [2]	Rat [2]
Typical adult wt, kg	70	454	454	228	73	0.55
Digestible calories, kcal	2590	14100	13800	8200	3800	68
Digestible protein, g	49	272	272	149	72.5	1.37
Protein/1000 kcal	19.0	19.3	19.7	18.1	19.1	19.5

[1] Computed from current Canadian dietary standard (23).
[2] Computed from NRC feeding standard (24).
[3] Computed from Brody-Kleiber equation (14, p. 136).

stores of the mother. If this is all demanded during the last third of pregnancy it is easily computed as (3 g × 6.25) 18.75 g of protein/day for this period. This would mean increasing the protein-to-calorie ratio in the diet during that period to 22 from 19 g/1000 kcal. However, there may be some doubt that this extra protein is normally advisable. It has been shown by Harris[1] that with range cattle extra fetal growth may be stimulated by supplementary dietary protein to the pregnant female, often resulting in difficult parturition sometimes fatal to the dam. Furthermore, omission of such supplement in no way prevents the young from reaching "normal" weight at weaning.

Modern feeding standards do not indicate any change in the *nutrient balance* of the ration during pregnancy for dairy cattle, beef cattle, swine, dogs or rats. In any case there is no evidence that, with normal dietaries, the possible slight drain on the body protein "reserves" of the mother to meet the full needs of the fetus are of measurable consequence. Platt (13) summed up the case for the human very concisely in the statement that, "An increase in the diet towards the end of pregnancy should be regarded as preparation for successful lactation. I think that is more to the point, perhaps, than finishing off the fetus." There is practical observation from all classes of farm mammals to support this view. Platt's remark is of some interest today when inhibition of lactation in some species seems to be of more concern than preparation for an abundant milk flow.

It is of interest also that, wherever any upward adjustment in allowance is made in the feeding of pregnant farm animals, it is made by increasing the quantity of the normal ration as a unit.

Lactation. The belief is commonly held that for lactation, dietary protein needs are increased to a greater extent than those for energy. A few simple calculations may clarify some of the facts as they are now accepted.

The efficiency of the energy of a normal diet for the production of milk energy is about 61% in the case of the cow, and this value has been applied to the human. On this basis, to produce 850 ml of human milk carrying 0.75 kcal/ml require (850 × 0.75) ÷ 0.61 = 1045 metabolizable dietary kcal.

If we assume an efficiency of dietary protein for human milk protein production of 75% (as it is with the cow), we can compute that to produce 100 ml of milk carrying 1 g of protein will require (1 × 100/0.75) = 1.33 g, and for 850 ml (1.33 × 8.50) = 11.3 g of dietary protein. Thus, for a daily (average) lactation of 850 ml the diet must supply 1045 kcal and 11.3 g of protein; or 10.8 g protein/1000 kcal of metabolizable energy. Some estimates place the dietary protein efficiency for human milk protein at 50%, in which case 16.3 g dietary protein/1000 kcal would be needed for human lactation. This is still less than the 19 g provided in the normal non-lactating diet. It is therefore probable that the normal maintenance diet supplying 19 g protein/1000 kcal needs no protein supplementation to make it adequate for lactation.

The milks of different mammalian species, however, differ in nutrient composition and hence require differing proportions of nutrients to energy for their production. For example, cow's milk carries 3.5 times more protein per liter than human milk, but only some 4% more energy. Consequently the ration digestible protein needed per 1000 digestible kcal (using the same ration efficiency as for the human) is about 3.6 times that for human milk (i.e., 39.0 g vs. 10.8 g). Table 2 shows rounded approximate values for 5 species.

There should be no change in the energy-to-protein ratio of the bovine diet for different levels of production (14, p. 325), the only adjustment being in the quantity of the ration fed. In the case of rats, dogs, sheep, and swine, where lactation is not measured directly, the adjustment of daily ration intake is either left to the nursing mother through ad libitum feeding, or is regulated by the feeder in accordance with the number of young in the nursing litter.

Incidentally, in modern practice the time of weaning from milk of calves and of baby pigs is often reduced from 30 to 5 and 60 to 15 days, respectively. Neverthe-

[1] Harris, L. E., Professor, Department of Animal Science, Utah State University, Logan, Utah. Personal communication.

TABLE 2
Grams digestible protein required per 1000 digestible dietary kcal to meet the specific needs of lactation

	g
Human	11.0
Cow	39.0
Sheep	35.0
Sow	35.0
Dog	55.0

less, with respect to early weaning from suckling, livestock practice has lagged behind that in the human family. Results of early weaning in general have been more rapid growth of the young than during comparable periods of nursing, and have made it clear that the nutritive properties of milk as an entire food for young mammals can be duplicated by other food combinations. The faster growth of early weaned young is, however, more often a reflection of fully adequate amounts of the diet, than of a more suitable balance of nutrients.

Quantitatively, the "normal" diet of the nursing mother should be increased daily to provide approximately 125 kcal extra for each 100 ml of milk produced. This amount of metabolizable energy (i.e., 125 × milliliters of milk produced per day) should be added to her caloric requirements for maintenance plus that appropriate to her physical activity.

Work (or other muscular activity). One further dietary category of adults — that of the physically active individual — remains to be dealt with, and is of importance with only 3 species: dogs, horses, and man. This category presents a unique situation, in that practice and classical theory are at sharp variance. Based on nitrogen balance studies, most of which were conducted on horses or men early in the century, nutritionists generally have been satisfied that physical exercise, per se, while requiring energy, does not demand any increase in protein intake over the maintenance level.

In 1963, however, Consolazio et al. (15) published the results of a 1960–61 N balance study, the data of which are of direct significance in this problem. In the report of these workers they comment pointedly that, "In general nitrogen balance losses have included only the urine and fecal excretions, although nitrogen losses are also observed in sweat and can possibly be found in expired air." They call attention to the fact that recent publications on human allowances have not considered the effect of these losses on nitrogen requirements. With respect to their observations in general, they state: ". . . there is an increase in sweat nitrogen with . . . an increase of physical activity . . ." even under conditions of fairly high protein intakes . . . the nitrogen balances were quite negative when the sweat losses were included." And again, ". . . the urinary and fecal nitrogen losses were remarkably constant signifying that the increased sweat nitrogen excretions are not compensated by decreases in the urine and feces."

Some of the data from this balance study can be used in computations pertinent to the subject under consideration in this paper. They are summarized in table 3.

TABLE 3
Some computations with respect to protein-kilocalorie requirements for work

Energy expenditure at imposed work	
50 min × 1.14 liters O_2 × 4.825 kcal/liter	= 338
50 min × 0.75 liters O_2 × 4.825 kcal/liter	= 171
Total	509
Equivalent digestible dietary kilocalories	= 549
Subsequent "daily" dermal protein loss [1]	
1273 mg N × 6.25 ÷ 1000	= 7.93 g
Equivalent replacement digestible dietary protein	= 10.5 g
Digestible protein required/1000 digestible kcal	
$\frac{10.5 \times 1000}{549}$	= 19.1 g

[1] Based on reported average excretion of nitrogen in sweat per 7.5-hour exposure period, comprised of two 50-minute periods of exercise on an ergometer and the remaining time of 5.8 hours at sedentary activity, in an environmental temperature of 21°C in one series and 47°C in a second.

These computations appear to support the premise that for nutritional equilibrium, physical activity requires protein as well as energy, and its *amount per 1000 kcal* of diet is about that of the normal maintenance ration. Hence the need can be met by an appropriate increase in the normal diet as a whole.

The use of nitrogen balance as the criterion of dietary protein requirement is complicated by the presence in the body of tissue (or cell) reserve protein which can be mobilized to "make good" current inadequate intake. Thus N equilibrium can be established at any level of protein intake above maintenance. In order, therefore, to demonstrate a protein requirement for exercise by N balance studies one must show either a greater output than intake of nitrogen, or a decrease in labile (reserve) tissue or cell nitrogen, or both, as a consequence of an imposed work load. Our own studies[2] lead us to believe that, depending on intake level, different tissues may, simultaneously, respond differently to exercise, in that blood serum albumin may decline while the gastrocnemius muscle N increases, and the skin remain unchanged in nitrogen content (as measured in these tissues, respectively, by albumin-to-globulin ratio; total N concentration; and RNA/DNA-to-total N/DNA ratios).

Direct estimation of the protein status of the body still awaits dependable methods, but ultimately, working adult animals consuming diets providing adequate energy for the work load involved, but restricted in protein intake to maintenance needs must show either negative nitrogen balance, or a loss in weight from depletion of body nitrogen reserves, or both, if nitrogen is in fact required for muscular exercise.

In practice it is not practical to increase the energy intake of horses or dogs at work, without also increasing the intake of protein. To provide horses with extra energy for work, addition of any suitable grain to the hay or hay-plus-grain maintenance diet results in an *increase* in the protein-to-energy ratio. For most dogs the working diet is simply more raw meat, or fish, or both, with the extra protein furnishing the needed additional energy.

With man, however, whose diet under usual living is put together in bits and pieces, with neither the parts nor their proportions ever the same either between comparable persons or for the same person at different meals or on different days, there is the possibility of incorporating essentially "empty calories" to meet the increased energy for work. If the maintenance diet is balanced to supply only the presumed maintenance protein requirement, then the addition of "empty calories" to meet the increased energy demands for work, results in a ration with a reduced energy-to-protein ratio, or as it is expressed by some, a wider nutritive ratio. With respect to such diets Mitchell (16) writes: "In practical nutrition, and in the absence of economic stringency or the unavailability of protein-rich foods, minimal protein nutrition is not in vogue; diets with the wide nutritive ratios associated with this type of nutrition are not palatable and are not selected from choice. Super-maintenance protein feeding during growth and muscular activity will provide the dispensable protein stores so advantageous when physiological adversity strikes. During muscular activity the increased caloric needs are commonly met by an increased consumption of the usual diet, not by the addition to this diet of non-nitrogenous items."

Most human foods when added singly to a balanced maintenance diet to supply "work" calories, result in altered ration balance through excesses of some nutrients, or shortages of others (including amino acids, minerals, and vitamins), or of both (table 4).

Deliberate diet alteration to satisfy the worker or athlete is usually accomplished by increased allowances of meat, fish, cheese, and to a lesser extent, of bread and potatoes. The former supply from 50 to 150, and the latter 20 to 30 g of protein/1000 digestible kcal, and their addition to the diet actually adds more protein relative to the energy than is called for in the maintenance diet. Most cakes and pastry furnish 10 g or more of protein/1000 kcal, and when combined with such foods as noted above, result in a final mixture that

[2] Unpublished.

TABLE 4

Nutrients per 1000 digestible kcal of the edible portion of the foods indicated (approximate values)

Nutrient	"Sedentary" diet	Sugar	Fats	Potatoes	Bread	Milk	Beef	Eggs	Fish
Digestible protein, g	19.0	0	1	20.0	31.0	51.0	60.0	78.0	160.0
Thiamine, mg	0.3	0	0	0.8	0.2	0.6	0.2	0.7	0.6
Riboflavin, mg	0.7	0	0	0.3	0.2	3.0	0.5	2.0	1.0
Iron, mg	3.0	0	0	0.1	3.0	1.0	8.0	2.0	5.0

carries at least the "normal" 19 g protein/1000 digestible kcal.

It appears, then, that recent studies support the view that physical exercise does in fact impose not only an energy requirement, but also a corresponding dietary protein requirement, and that in amount the latter is in the same ratio to the former as it is in the properly balanced maintenance diet. This view is consistent with a statement in the report to the FAO Committee on Protein Requirements (17) that, "Increase in muscle mass associated with athletic training and with seasonal increase in muscular activity creates a need for protein in addition to the average minimum requirement." (and) ". . . in such circumstances the reference requirement for the group undertaking such heavy work should be substantially raised."

The question of whether muscular activity per se requires protein is largely academic in areas where protein-rich foods are economical and abundant, in the sense that the practical feeding of "working" individuals almost always results in the addition of adequate protein incidental to providing the needed extra calories. This in no way justifies ignoring the evidence or specifically indicating in feeding or dietary standards that physical activity does not require protein expenditure over that of sedentary living. Dietary standards (as distinct from recommended allowance tables) are (or should be) scientific documents intended to give minimal nutrient requirements compatible with health and performance of the individual specified. As such they serve an important function in guiding agencies (usually governmental) which must provide food or regulate its distribution under conditions of emergency or disaster where restriction or rationing must be imposed.

Growing animals

In the case of growing individuals, where appreciable positive N, Ca, and P balances are necessary, the acceptable diet must carry an increased concentration of these nutrients. Since juveniles are also more active than sedentary-living adults, the growing-period energy allowances are also larger per unit of body weight, than for adults. Many nutrients, however, should remain in fixed ratios to energy, since they are primarily needed to metabolize energy.

The actual dietary demands for protein relative to energy by the growing boy or girl are not as great as is popularly supposed. Terroine (18) calls attention to the error in the common belief that ". . . the whole of the physiology of the child is dominated by the need for protein synthesis, and that they are, as it were, factories for the intensive production of such proteins." His data show that the net growth requirement for protein "rarely exceeds 2 grams per day." Hegsted's (19) calculations agree with Terroine's data, and he also emphasizes "the relatively small contribution that growth makes to the protein need of the child after the first few months of life."

Using the assumption that the true maintenance protein requirement is 12.5 g/1000 *basal* kcal, and that gains in weight are 18% protein, Hegsted (19) observed that the total daily *net protein* requirement of children was essentially constant at about 14 g/1000 *basal* kcal. To check the latter, Mitchell (16) used data from other sources but similar assumptions, and predicted daily gains. He found an average of 13.8 g protein/day per 1000 *basal* kcal, but a slightly greater tendency for higher protein in the first 2 years of age. He concludes that, ". . . regardless of sex, rate of growth, and age, between 1 and 2

years and early maturity, the total *net protein* requirement for maintenance plus growth varies with the basal metabolism of energy so that it amounts to 13–15 grams per 1000 *basal calories* per day." His tabulation shows values for 16.3 and 14.6 g protein for ages 1 and 2 years; and 13.9 to 12.9 for ages 3 to 19 years, respectively.

Insofar as digestible protein intake in relation to digestible energy need is concerned, it is, with all species, a continuously declining variable following the true growth rate of the juvenile in question. The general pattern can be seen by plotting the somewhat comparable data for digestible protein required per 1000 digestible kcal for children and for market pigs against successive weights, the weights expressed for each species as the percentage of the adult metabolic size attained. The limited data available from the latest feeding standards thus plotted, and the regressions fitted by inspection, are shown in figure 1. This chart reflects clearly the uniquely long juvenile period in the human species.

From the practical standpoint it is neither feasible, nor necessary, to adjust daily the nutrient-to-calorie ratios for growing individuals. In pig feeding the growing period is divided into 3 "ration" periods: 4.5 to 18 kg; 18 to 55 kg; 55 to 91 kg, during which the protein per 1000 kcal is successively changed from 37 g to 30 g to 25 g. The 4 "steps" of change in ration balance are shown on the graph; and suggested comparable steps for the human are marked in accordance with the position of the appropriate plotting points. These latter suggest 3 "growing" rations, with changes of protein concentration per 1000 digestible kcal from: 28 g to 23 g to 21 g to the adult 19 g, to be introduced when 22, 35, and 72%, respectively, of adult metabolic weight has been attained.

Quality of protein

In the above discussions of protein-energy balance, no mention has been made of the effect of quality of protein on the daily amounts required for adequacy in any given case. This factor must be considered, however, because as the biological value of the protein declines, increasing quantities must be ingested to provide retention of the amounts of the essential amino acids needed. It is convenient to consider the dietary protein complex of North Americans as excellent, good, or average, corresponding to biological values (BV) of 100% (animal + marine), 75% (1 animal + 1 marine + 1 plant), and

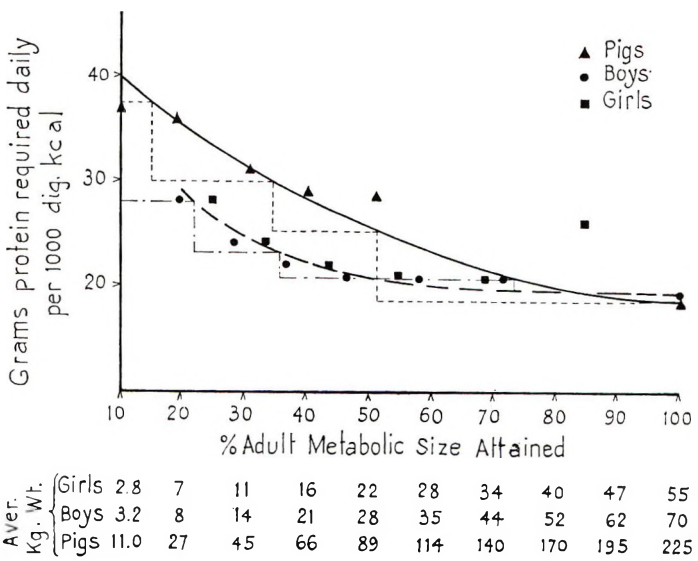

Fig. 1 Grams digestible protein required by juveniles per day per 1000 digestible calories.

50% (1 animal + 1 marine + 2 plant). The equivalent intakes of these 3 categories are not exactly known; but Leitch and Duckworth (20) reported daily intakes of 52 g of protein of *average* quality gave equal chances of positive or negative N balance with adults. The Princeton Conference (21) concluded that 32.5 g was adequate where quality was *good*, and the FAO Committee on Protein Requirements (17) reported that 24.5 g of excellent quality protein was adequate to maintain N equilibrium with adults.

To specify, then, the minimal protein intake required daily or per 1000 kcal, its BV must be known or estimated. The 3 minimums (24.5, 32.5, 52.0) represent increases in necessary intakes of the order of 100: 132: 210%, to correspond to declining BV of 100: 75: 50%. Since a 70-kg adult human at light work (550 kcal for work) requires about 2800 metabolizable kcal daily, minimal protein requirements per 1000 kcal could be computed for 3 qualities as: BV 100% = 8.8 g; BV 75% = 11.6 g; BV 50% = 18.5 g.

Inter-nutrient balances

The problems of balance between amino acids, between mineral elements, and between vitamins, to say nothing of intergroup balances and their ratios to energy, are under active experimental study for many species. To date no data are available which indicate their optimal proportions in the human diet, except for the FAO (17) pattern of amino acids. It becomes necessary, therefore, for the present to assume that the minimal amounts of nutrients needed daily by a specified individual, as the reference man of the NRC Food and Nutrition Board recommended allowances (22), represents a working balance pattern between whatever nutrients we are to include in a human dietary standard.

We must also make assumptions, in almost all cases, regarding the caloric intake of human subjects on whose performance the nutrient requirements have been based, for this has seldom been reported. The subjects have been described as to sex, weight, and activity, but not often quantitatively as to energy intake. From the descriptions offered it is probable that most trials involve subjects who could be classed as 70-kg males at very light "work," such as young adult medical students. For them we can assume a daily metabolizable energy need of 2250 kcal for maintenance plus about 550 kcal for activity in excess of sedentary living. This estimate of daily intake of 2800 kcal is realistic enough to serve, in this proposal, as a working base for computing the nutrients required by adults per 1000 kcal of metabolizable dietary energy.

Energy,[3] the least common denominator for the dietary standard

Nutrient and energy needs of individuals or groups of individuals for whom the same "balance" in their rations is optimum, are ultimately determined by feeding trials or other experimentation. As an example, assume that from the published results of such tests an investigator assembles evidence that the minimal requirement for healthy 70-kg male adults, at light work (550 kcal) is, on the average: energy, metabolizable, 2800 kcal; protein (good quality), 32.5 g; calcium, 450 mg; thiamine, 0.56 mg. In accordance with present thinking, other adults, heavier or more active, or both, but otherwise comparable, will require more of each of these diet components, but with the same inter-nutrient and nutrient-energy balance. The tabulation of the needs of such groups can be generalized if the nutrients are expressed in amounts per 1000 kcal, as: energy, 1000 kcal; protein, 11.6 g; calcium, 160 mg; thiamine, 0.2 mg.

To arrive at the minimal daily nutrient requirements for a person it is necessary to ascertain his daily caloric needs, and to compute for each nutrient:

$$\text{Nutrient/1000 kcal} \times \frac{\text{daily kcal required}}{1000}.$$

For purposes of dietary standards, the daily energy needs may be estimated for adult individuals from their weights. For purposes of this discussion we shall use the general equation (11):

For maintenance, kcal 24 hr = $93(W_{kg}^{0.75})$

For "light" work, kcal 24 hr = $23(W_{kg}^{0.75})$

For average work, kcal 24 hr = $55(W_{kg}^{0.75})$

[3] Since the energy requirements of man and the energy of his foods and diets is universally expressed in terms of metabolizable kilocalories, this category of energy will be used in the remainder of this review.

From these equations it is possible to compute the daily caloric needs of a 70-kg man at average work, or of an 80-kg man at light work, as in table 5.

To obtain the 24-hour dietary requirements for these men, the calories and nutrients per 1000 kcal are multiplied by 3.58 and 3.1 for the 70- and 80-kg adults, respectively.

If, in addition to a statement of minimal requirement, a guide to daily nutrient *allowances* is also wanted, the *requirement* values may be increased systematically by an appropriate factor. For example, the standard deviations of voluntary food intake under unrestricted allowances is of the order of 16% of the average for domestic farm animals fed identical rations and differing in live weight by 10% or less. It is known that over extended periods, mature farm animals and adult humans tend to consume their respective diets in amounts that maintain a steady body weight. Until data are available, it might be assumed that the variability in voluntary caloric intake of adult humans of comparable weight might also be of the order of 16% of their average energy requirement. On this basis increasing average nutrient requirements relative to energy by 50% would "insure" adequate allowance of nutrients for 599 out of 600 individuals. The single exception probably should be considered a diet therapy case. In general no nutritional benefit can be expected, and risk of undesirable results might be incurred by continued intake of some nutrients in excess of 50% over need. However, increases in the total diet without change of total nutrient-energy balance are largely innocuous except where problems of overweight are involved.

To return to our example, the final values for the 70- and 80-kg men are shown in table 6.

The necessity for computing specific caloric needs by formula can be avoided by preparing a regression chart with weights along the axis and using multiple ordinate scales to accommodate categories in addition to sedentary living that contribute to the total caloric need, but must be independently determined. Figure 2 is such a chart for humans. It includes juveniles, and 3 categories of work intensity as well as adult "sedentary" living.

The number of different tabulations of nutrients necessary in a complete dietary or feeding standard will correspond to the number of rations requiring different nutrient balance. For man there appear to be at least four: one for adults, three for

TABLE 5

Daily caloric requirements of two adult men

Description	Sedentary living	Light work	Average work	Total 24 hr
	kcal	*kcal*	*kcal*	*kcal*
70-kg Man, average work	2250 [1]		1330	3580
80-kg Man, light work	2485	615		3100

[1] All numbers rounded to practical values.

TABLE 6

Example of tabulation of dietary requirements and the maximal recommended allowances

Energy and nutrients	70-kg man, average work		80-kg man, light work	
	Required	Upper limit of the justifiable range [1]	Required	Upper limit of the justifiable range
Energy, metabolizable kcal	3580		3100	
Protein, g BV [2] 100%	31.5	47.2	26.0	39.0
BV 75%	41.5	62.3	34.2	51.0
BV 50%	62.0	93.0	51.2	77.0
Calcium, mg	572	858	472	706
Thiamine, mg	0.72	1.08	0.59	0.89

[1] See text, page 362.
[2] BV indicates biological value.

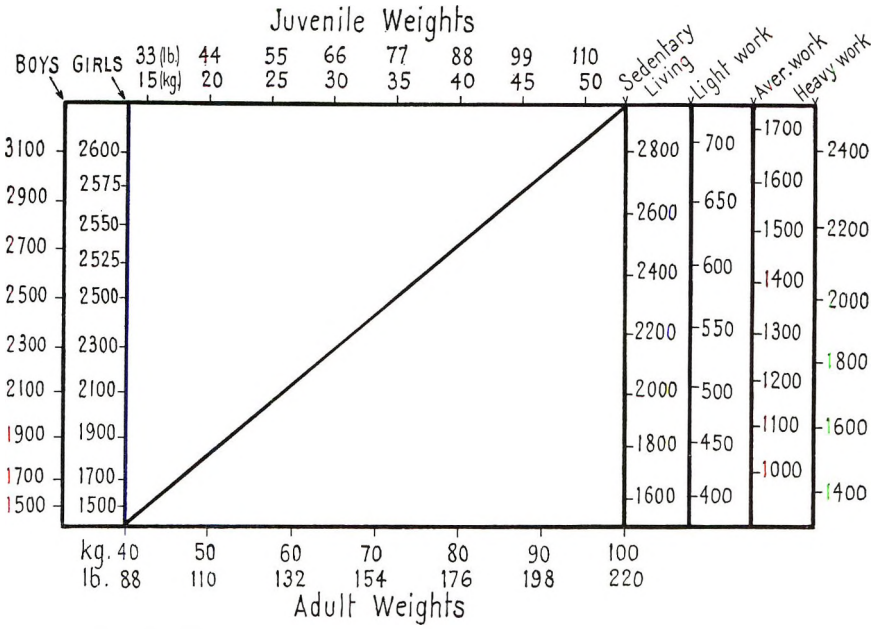

Fig. 2 Daily metabolizable calorie requirements, human species.

juveniles, plus one (optional) for adults to show the upper limits nutritionally justified (i.e., above which no nutritional advantage can be expected).[4]

In the case of juveniles in each of three feeding categories no change in nutrient concentration in the dietary energy is warranted with the present meager knowledge of requirements.

Table 7 illustrates the tabulation of daily human nutrient requirements and the maximal limits justifiable per 1000 kcal. (The data are examples only and imply no official acceptance as requirements.)

To convert these tabulations to daily nutrient needs requires only the figures for the necessary daily energy intake, as from figure 2. As read from figure 2 the needs of juveniles, and for adults at sedentary living are obtained directly as single values; but for adults at work, a second reading from the appropriate "work scale" is necessary, and the 2 values (sedentary and working) added to obtain the total day's requirement. The change in energy requirements shown between boys and girls is not universally agreed on, but is incorporated here to illustrate the possibility of its inclusion in this method.

As an example, assume a family group of:

Man	70 kg	average work
Wife	60 kg	light work
Son	30 kg	normally active
Daughter	16 kg	normally active

From figure 2 we determine the energy requirements to be:

Man	2250 + 1330 = 3580 kcal
Wife	2010 + 500 = 2510 kcal
Son	2275 kcal
Daughter	1550 kcal

To obtain the daily dietary nutrient requirements the amount of the several nutrients tabulated in the column of table 7 for adults are multiplied by 3.58 and 2.51 for the man and wife, respectively; and by 2.275 and 1.55 for the son and daughter, respectively.

In summary, it appears to this author that there is valid evidence to justify the conclusion that energy intake directly or indirectly "determines" for most nutrients the intake that is compatible with maximal efficiency of the diet as a whole in the maintenance and productive performance of the animal body.

The method of computing the desirable nutrient makeup of diets illustrated facilitates maintenance of the same "intra-

[4] See page 362, column 2.

TABLE 7

Proposed scheme of tabulating dietary standards for humans; daily nutrient requirements and allowances per 1000 kcal, metabolizable energy

Nutrients	Adults		Juveniles Recommended allowances		
	Minimal requirement	Upper limit of justifiable range [1]	to 10 kg	10–20 kg	20–70 kg (adult)
Protein, g					
BV [2] 100%	8.8	13.2	28	23	19
75%	11.6	17.4	37	30	25
50%	18.5	27.8	—	—	—
Edible fats, g	—	26.0	26	26	26
Calcium, mg	160	240.0	625	500	350
Phosphorus, mg	160	240.0	625	500	350
Iron, mg	1.1	1.7	6	3	4
Thiamine, mg	0.2	0.3	0.2	0.2	0.2
Riboflavin, mg	0.3	0.45	0.3	0.3	0.3
Niacin, mg	1.0	1.5	1.0	1.0	1.0
Ascorbic acid, mg	3.6	5.4	25	15	10

[1] I.e., intakes above this limit serve no necessary or useful nutritional purpose, and may produce undesirable results.
[2] BV indicates biological value.

group" balance between nutrients, as well as between energy and the nutrient groups as a whole, in rations required in different amounts daily to meet size or performance differences, or both, of the individuals of the same diet category. Tabulation of the complete "standard" is also greatly simplified and condensed without sacrificing necessary or desirable detail. Expansion to include further nutrients or additional dietary groups, or both, is practicable if and where such may be found desirable as a consequence of new facts on requirements or to meet special circumstances.

Finally, the plan is applicable to all species for which feeding standards are prepared, and the form of tabulation facilitates recognition of similarities in comparative nutrition.

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Anti-rachitic Effects of Soybean Preparations for Turkey Poults[1]

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ABSTRACT Broad Breasted Bronze turkey poults fed to 4 weeks of age a glucose-isolated soybean protein diet, supposedly adequate in all known nutrients, developed severe rickets and grew poorly. Heated soybean meal, raw soybean meal, or water extracts of raw or heated soybean meal partially prevented the rickets and markedly improved growth. A several-fold increase in vitamin D supplementation effected similar, but submaximal responses. Raw or heated soybean meal also appeared to contain a factor(s) required for maximal growth and bone calcification of turkey poults, even when the level of vitamin D_3 was greatly increased.

Numerous published reports have indicated that soybean meal contains an unidentified growth factor (UGF) for young chicks and poults. Hill (1) reported that soybean meals of various sources differed in their growth-promoting ability and attributed this to variations in their UGF content. Kratzer and co-workers (2, 3) reported that a methanol extract of soybean meal gave marked growth responses when added to an isolated soybean protein-glucose-type diet. Wilcox and associates (4, 5) noted that a water extract of soybean meal (WE) added to a diet similar to that used by Kratzer et al. improved growth of turkey poults. The basal diet resulted in very poor growth, and replacement of a portion of the isolated soy protein with soybean meal, improved growth. Their results suggested the involvement of more than one factor, as submaximal responses were obtained with the ash and a nondialyzable fraction of WE.

In further studies on growth-promoting properties of water extracts of soybean meal at this laboratory, rickets developed in poults fed the basal diets, and addition of soybean meal fractions prevented development of rickets. Some of the experiments conducted on the anti-rachitic properties of soybean meal and water-soluble fractions thereof are described.

MATERIALS AND METHODS

The basal diet used in the following experiments was changed only slightly from that of Wilcox et al. (4) and is described in table 1. The high level of soybean protein, together with the methionine hydroxy analogue and glycine, gives a calculated protein content of approximately 39%. A commercial grade of highly available dicalcium phosphate[3] was used in place of the

TABLE 1
Composition of poult basal diet

	%
Isolated soybean protein[1]	44.0
Glucose monohydrate	45.0
Corn oil	2.0
Dicalcium phosphate [2]	4.0
Limestone [2]	1.5
Methionine hydroxy analogue	0.7
Glycine	0.5
Minerals,[2] vitamins [3] and antibiotics [4]	2.3

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.

[2] Total added minerals were: (in %) Ca, 1.52; P, 0.72; K (KCl), 0.60; Na (NaCl), 0.20; and Cl, (KCl and NaCl), 0.54; and (in ppm) Mg ($MgCO_3$), 580; Zn ($ZnCO_3$), 145; Mn ($MnSO_4$, 70%), 65; Fe ($FeSO_4$), 89; Cu ($CuSO_4$), 23; B (H_3BO_3), 20; Br (KBr), 16; Al [$K_2Al_2(SO_4)_4$], 15; Co ($CoCl_2 \cdot 6H_2O$), 10; Si ($Na_2Si_4O_9$), 5; Mo ($NaMoO_4 \cdot 2H_2O$), 4; I (KI), 3; Se (H_2SeO_3), 2.

[3] Total added vitamins were: (per kg) vitamin A, 15,000 IU; vitamin D_3, 2,000 ICU; vitamin E, 290 IU; (replaced with 98 IU vitamin E and 222 mg ethoxyquin after experiment 3); choline, 2.97 g; (in mg) *i*-inositol, 1100; *p*-aminobenzoic acid, 110; niacin, 100; Ca pantothenate, 44; thiamine, 22; riboflavin, 22; pyridoxine, 22; ascorbic acid, 22; menadione sodium bisulfite, 8.8; folic acid, 8.8; and (in μg) biotin, 440; vitamin B_{12}, 33.

[4] Oxytetracycline, 33 mg/kg.

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[2] On leave from South Dakota State College, Brookings, South Dakota.

[3] Dynafos, International Minerals and Chemicals Corporation, Skokie, Illinois.

C.P. dicalcium phosphate used by Wilcox et al. (4). Other modifications were primarily limited to the mineral mix and included the following: use of carbonate salts of magnesium and zinc in place of sulfate and chloride salts, selenous acid in place of sodium selenite, a reduction of iodine, and increase in molybdenum, cobalt, aluminum, magnesium and potassium, and the addition of potassium bromide. Sodium arsanilate was deleted and replaced with oxytetracycline. All of the known essential minerals were supplied in adequate quantity, and the salts used were readily soluble. All vitamins were provided at 2 to 5 times the recognized requirement levels suggested by the NRS (6).

Broad Breasted Bronze turkey poults (not sexed) were fed the basal diet to one week of age. Poults were then distributed within battery brooders into groups of 8 to 12 with 3 groups per treatment and fed for a 3-week experimental period. Group weights were obtained initially and at weekly intervals. After termination of the experiment, the left tibias of 5 turkeys per group were removed and cleaned and bone ash was determined by the AOAC method (7). Statistical analyses were conducted according to the procedure of Duncan (8).

In the first experiment, a 30% protein diet was also prepared by replacing 12% of the soybean protein with glucose monohydrate. Previous studies by Guenthner[4] had suggested the need for investigating the protein level required for maximal response to the WE. In subsequent experiments, only the 39% protein basal diet was used. The WE was prepared according to the method of Wilcox et al. (4) from both heated commercial (SBM) and raw (RSBM) soybean meals (WE and WER, respectively). This process involved 3 extractions of ca. 10 kg 50% soybean meal (raw or heated) with ca. 10 liters tap water at pH 4.7. The combined extracts were concentrated in a vacuum condenser at 70 ± 10°C to ca. 20 to 30% solids. On a dry weight basis, 1, 2, and 4% WE were added to both the 30 and 39% protein diets in experiment 1, to determine optimal levels of supplementation. These levels were essentially equivalent initially to 5, 10 and 20% soybean meal. For the 2% level, the WE had been concentrated to dryness and added as a hygroscopic brown powder. All other WE or WER supplements in this or subsequent experiments were added as a brown syrup-like liquid. For the last treatment of experiment 2 and for experiments 4, 5, 6 and 7 the extraction procedure was altered in that the initial extraction was accomplished at pH 6.8 followed by adjusting the pH to 4.7 and discarding the precipitate.

EXPERIMENTAL RESULTS

The body weight and bone ash data obtained in experiment 1 are shown in table 2. Rickets was observed in poults fed each of the basal diets and was partially prevented by addition of 2 and 4% WE. The WE did not increase bone ash to a level of 46 to 47%, which is the usual range of bone ash values obtained with practical diets in this laboratory. Although both basal diets supported similar rates of growth, bone ash was significantly depressed by the higher protein level. Statistically significant improvements in growth and bone ash were not obtained by adding WE to the lower protein diet, although it appears that growth was improved by 4% WE. However, marked and significant responses were obtained with the higher protein diet at both the 2 and 4% levels of WE. Tibias from the basal groups were rubbery and severely deformed, typical of a vitamin D deficiency.

TABLE 2

Effect of water extract of soybean meal and dietary protein level on growth and bone ash of turkey poults (experiment 1)

Protein level	Water extract supplement [1]	4-Week avg wt	Bone ash [2]
%		g	%
30	None	437 [ab 2]	38.3 [bc 3]
	+1%	438 [ab]	41.1 [c]
	+2%	463 [ab]	42.2 [c]
	+4%	495 [bc]	40.6 [c]
39	None	422 [a]	33.7 [a]
	+1%	435 [ab]	35.3 [ab]
	+2%	538 [c]	40.0 [c]
	+4%	531 [c]	41.3 [c]

[1] From heated soybean meal; 1% on a dry weight basis was equivalent to 5% soybean meal.
[2] Percentage of dry, defatted bone.
[3] Values followed by the same letters show no significant difference at the 5% level of probability.

[4] Personal communication, South Dakota State College, 1962.

In a repetition of this experiment, rickets and poor growth were again obtained with the 30 and 39% protein basal, and 2 and 4% WE partially prevented rickets and greatly improved growth. A subsequent experiment was designed to obtain information on the activity of various soybean fractions.

Data obtained from experiment 2 (table 3) indicate that the factor(s) in SBM that prevented rickets and promoted growth was either more concentrated in or more easily extracted from raw soybean meal than heated meal. The factor(s) was apparently not specifically associated with protein, as the extract at pH 4.7, where most of the protein is insoluble, was as effective as a pH 6.8 extract, where much of the protein is soluble. The WE may not contain all of the anti-rachitic activity of SBM because on an equivalent basis it appeared to be less active than SBM.

TABLE 3

Effect of soybean meal and water extracts on poult growth and bone ash (experiment 2)

Supplement [1]	4-Week avg wt	Bone ash [2]
	g	%
None	385 [a 3]	30.9 [a 3]
5% SBM [4]	476 [bc]	35.7 [b]
1% WE [5] (≏ 5% SBM)	439 [ab]	33.2 [ab]
1% WER [6] (pH 4.7)	542 [c]	39.6 [c]
1% WER (pH 6.8)	511 [bc]	41.4 [c]

[1] All diets contained vitamin D_3 at 2000 ICU/kg and 39% protein.
[2] Percentage of dry defatted bone.
[3] Values followed by the same letters show no significant difference at the 5% level of probability.
[4] Indicates heated soybean meal.
[5] Indicates water extract of heated soybean meal.
[6] Indicates water extract of raw soybean meal, ≏ 5% raw soybean meal.

Following these studies, particular attention was given to the potency of vitamin D_3 supplements. Chemical assays for vitamin D by 2 commercial laboratories,[5,6] a biological assay by one of the laboratories[5] and a chick study conducted in this laboratory showed the supplements contained within 15% of their stated potency. Additional amounts of dicalcium phosphate and manganese were tested. The results obtained in 4 experiments with these supplements, SBM and WER, are shown in table 4. Only where vitamin D_3 was reduced to 880 ICU/kg was there a highly significant response, although with the higher levels of vitamin D, SBM gave improved bone ash ($P < 0.05$) and WER gave a growth response ($P < 0.05$). On the other hand, additional manganese or dicalcium phosphate had no statistically significant effect upon growth or bone ash. Poults fed the

[5] Dawes Laboratories, Chicago.
[6] Hoffmann-La Roche, Inc., Nutley, New Jersey.

TABLE 4

Effect of minerals, soybean meal and water extract of raw soybean meal on growth and bone ash (experiments 3, 4, 5 and 6)

Exp. no.	Vitamin D_3 level [1]	Supplement	4-Week avg wt	Bone ash [2]
	ICU/kg		*g*	%
3	2160	Basal	436 [a 3]	33.9 [a 3]
		Mn, 65 ppm	418 [a]	36.1 [ab]
		SBM,[4] 5%	474 [a]	37.5 [b]
		Mn + SBM	481 [a]	36.8 [ab]
4	2390	Basal	482 [a]	39.5 [a]
		Dicalcium phosphate, 1%	479 [a]	41.8 [a]
		WER,[5] 1% (≏ 5% RSBM)[6]	538 [b]	41.6 [a]
5	0	Basal	— [7]	24.0 [a]
		WER, 1%	— [7]	24.4 [a]
6	880	Basal	414 [A]	31.0 [A]
		WER, 1%	506 [B]	40.7 [B]

[1] Total vitamin D_3 content of diets; all diets contained 39% protein.
[2] Percentage of dry, defatted bone.
[3] Values followed by the same small letters within an experiment show no significant difference at the 5% level of probability; those followed by unlike capital letters show significance at the 1% level of probability.
[4] Indicates heated soybean meal.
[5] Indicates water extract of raw soybean meal.
[6] Indicates raw soybean meal.
[7] All poults died before termination of experiment; bones saved for ashing from poults dying between 3 and 4 weeks of age.

vitamin D-free basal diet and those fed the basal diet plus 1% WER diets died before the termination of experiment 5 and no differences in growth were observed prior to death. The bone ash values for 3-week-old poults were markedly similar, indicating that the 1% WER contained no vitamin D activity per se.

Subsequently, it was deemed desirable to determine whether soybean supplements would have any effect on the requirement for vitamin D. An incomplete factorial study (experiment 7) was conducted using combinations of isolated soybean protein and 5% raw soybean meal, 30% heated soybean meal and graded levels of vitamin D_3. The diets were made isonitrogenous. A preliminary study had shown 5% raw soybean meal to be deleterious to growth and bone ash at the 880 ICU level of vitamin D. Therefore, for this treatment the low level of vitamin D was replaced by a level 2 times higher than that used in the other treatments. The data from this experiment (table 5) show that even 3520 ICU of vitamin D_3 did not permit maximal growth when the birds were fed the basal diet. On the other hand, 1760 units were adequate for maximal growth with the supplemented diets. For bone ash, 1760 and 880 units were adequate for the maximal significant effect with the basal and 30% SBM-supplemented diets, respectively. However, a trend toward increased bone ash values with higher levels of vitamin D_3 was evident. No significant responses to vitamin D in either growth or bone ash were obtained with the raw soybean meal-supplemented diets.

DISCUSSION

Scott et al. (9) showed that soybean meal contains a factor that aids in utilization of anhydrous dicalcium phosphate. Their work, together with the data presented here, demonstrates further the complexity of nutritional factors involved in bone formation, as well as the nutritional factors in soybean meal. Whether the factor(s) involved in phosphate and vitamin D utilization are identical cannot be ascertained from present information.

The lack of significant growth or calcification responses to the WE or SBM supplements in several of the experiments with similar levels of vitamin D_3 was due in part to the higher level of the vitamin used and probably also to the greater stores of the vitamin carried by the poults when placed on dietary treatments. Variations in body stores of the vitamin as well as the anti-rachitic factor could account for the variation in responses obtained on the basal and similarly supplemented diets.

No vitamin D activity was demonstrated in the water extract of soybean meal by adding the extract to a diet unsupplemented with vitamin D, but marked responses in growth and bone ash were obtained from 1% WER or from 30% SBM in the presence of 880 ICU of vitamin D/kg. Therefore, the available information suggests that the anti-rachitic factor does not replace a minimal need for vitamin D,

TABLE 5

Effect of heated and raw soybean meal on growth and bone ash response to different levels of vitamin D (experiment 7)

Vitamin D_3 level	Dietary treatment	Avg 4-week wt	Bone ash [1]
IU/kg		*g*	*%*
800	Basal [2]	382 [a 3]	33.6 [a 3]
1760	Basal	449 [b]	37.6 [b]
3520	Basal	510 [bc]	40.4 [bc]
880	30% SBM [4]	534 [cd]	44.0 [de]
1760	30% SBM	597 [de]	46.1 [e]
3520	30% SBM	627 [e]	46.7 [e]
1760	5% RSBM [5]	549 [cd]	42.6 [cd]
3520	5% RSBM	588 [de]	44.2 [de]
7040	5% RSBM	575 [de]	44.7 [de]

[1] Percentage of dry, defatted bone.
[2] Isolated soybean protein; all diets contained 39% protein.
[3] Values followed by the same letters show no significant difference at the 5% level of probability.
[4] Indicates heated soybean meal.
[5] Indicates raw soybean meal.

but that the factor either improves the metabolic efficiency of vitamin D or prevents its binding or destruction in the diet. Whether this effect occurs before or after consumption of the diet has not been investigated. The detrimental effect of the higher level of isolated soybean protein, together with a lessening of the detrimental effect by replacing some of the protein with the soybean meal, suggests that the protein supplement per se is responsible for the much higher vitamin D requirement. This aspect of the problem is being investigated further.

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Carbohydrate and Fat Metabolism and Response to Insulin in Vitamin B_6-deficient Rats[1]

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ABSTRACT Vitamin B_6-deficient rats, compared with appropriate controls, showed growth inhibition, and a decrease in total carcass lipids. The palmitoleic acid content of the liver and epididymal fat pad lipid decreased and the content of stearic and linoleic acids increased. All of these changes were partially prevented by insulin administration. The deficient animals showed hypoglycemia and a decreased ability to clear glucose from the blood after the administration of glucose, and decreased tolerance to insulin. "Insulin-like activity," determined by the effect on epididymal fat pads, of the serum and pancreatic tissue was lower than in control animals. The epididymal fat pads of deficient animals were much more sensitive to insulin than those of controls as measured by net gas exchange and incorporation of labeled glucose into fat.

A nearly universal effect of feeding nutritionally inadequate diets is decrease in food intake accompanied by inhibition of growth. Perennial problems complicating the study of nutritional deficiencies have been how to explain the mechanisms causing the change in food intake and to distinguish between the direct effects of the lack of the nutrient being studied and those effects that are the consequence of decreased food consumption. In vitamin B_6 deficiency, for example, changes occur which may not be the direct consequence of a lack of vitamin B_6-containing enzymes, but may be due to decreased food intake. On a vitamin B_6-deficient regimen, the rat shows growth retardation (1), decreased body fat (2, 3), decreased efficiency of food utilization (4), lowered blood glucose and liver glycogen (5), and increased stearic acid and decreased palmitoleic acid in its total fatty acid content (6). Beaton et al. (7) have reported that insulin injections of vitamin B_6-deficient rats, can to a considerable extent, prevent the inanition and decreased fat storage in this deficiency. Since Kuno (8) reported degenerative changes in the β cells in the Islets of Langerhans in vitamin B_6 deficiency, it appeared possible that the changes in carbohydrate or fat metabolism in vitamin B_6-deficient rats might be due to changes in availability of insulin. In this paper, further evidence will be presented to show that many of the changes associated with vitamin B_6 deficiency in rats can be reversed by insulin administration, that in vitamin B_6-deficient rats glucose tolerance is decreased, that the insulin-like activity of their sera and pancreata is decreased, and that the sensitivity of their epididymal fat pads to insulin is increased.

EXPERIMENTAL AND RESULTS

Male Charles River CD rats, 3 or 4 weeks old, were used. They were maintained with purified diets fed ad libitum, unless otherwise stated, for varying periods of time. The basal diet contained: (in per cent) casein, 15; sucrose, 75.7; cod liver oil, 1.0; corn oil, 4.0; salts IV (9), 4.0; and choline chloride, 0.3. Vitamins were added so that each 100 g of diet contained 0.4 mg thiamine, 0.8 mg riboflavin, 4 mg niacin, 0.02 mg biotin, 2 mg calcium pantothenate, 0.1 mg folic acid, 0.1 mg menadione, 0.005 mg vitamin B_{12}. The control groups received, in addition, 1 mg pyridoxine·HCl/100 g of diet. In one experiment, the 4% corn oil of the basal diet was replaced by 4% trimyristin. In another experiment, a high fat diet was used that contained 42% hydrogenated shortening. This diet was formulated so that it contained the same amount of pro-

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tein, salts, and vitamins per calorie as the basal diet.

Body composition determinations were made on chloroformed rats whose gastrointestinal tracts were first washed and blotted. The carcasses were autoclaved in Mason jars for one hour at 115°C. These were then homogenized in a Waring Blendor and dried to constant weight. Total lipids were determined on aliquots of the dry solid according to Folch et al. (10). The fatty acid composition was determined by use of gas-liquid chromatography (11). Ash was determined using the method of Mickelsen and Anderson (12), and nitrogen by the procedure of Natelson (13). Tissue vitamin B_6 analyses were estimated by the microbiological method of Atkin et al. (14), after hydrolysis of the samples in 0.055 N H_2SO_4 at 112° for one hour. Serum and blood glucose were determined by the alkaline copper method of Nelson (15). Insulin-like activity was determined by two methods, both based on the response of the rat epididymal fat pad to insulin. The procedure of Renold et al. (16) in which the liberation of radioactive CO_2 from glucose-1-C^{14} is measured was used, or the net gas exchange was measured according to Ball et al. (17). In these studies made in Warburg flasks, Krebs-Ringer bicarbonate buffer, pH 7.4, containing 50 mg of gelatin/100 ml and 300 mg of glucose/100 ml was used. The gas phase was a mixture of 5% CO_2-95% O_2. For each assay by either procedure, standard curves were obtained by using standards of 30 to 500 μunits of glucagon-free insulin,[2] and from the curves the amounts of insulin-like activity in the samples were estimated.

Effect of insulin treatment on vitamin B_6-deficient rats. Groups of 4-week-old rats were fed the basal diet with and without vitamin B_6 for 4 weeks. The animals in two of the vitamin-deficient groups received daily intraperitoneal injections of either 1 or 4 units of protamine-Zn-insulin[3] during the last 3 weeks of the experiment. Prior to killing with chloroform, 0.1 ml of blood was drawn from the tail veins and immediately following death approximately 50 mg of liver and 50 mg of kidney were removed for vitamin B_6 assay. The composition of the rest of the carcass was then determined as described above.

The results of this experiment presented in table 1 indicate that the growth retardation observed in vitamin B_6-deficient rats was largely overcome by 4 units of insulin per day during the 3 weeks this treatment was used ($P < 0.001$). This effect was accompanied by a marked increase in food

[2] Obtained from Eli Lilly and Company, Indianapolis.
[3] See footnote 2.

TABLE 1

Effect of insulin on body weight, body composition and vitamin B_6 levels

	Diet			
	With vitamin B_6	Without vitamin B_6	Without vitamin B_6	Without vitamin B_6
Units of insulin/day	0	0	1	4
No. of rats	6	11	8	6
Final body wt, g	130 ± 4.5 [1]	91 ± 3.4	101 ± 5.6	116 ± 5.2
Tail length, cm	15.2 ± 0.96	14.0 ± 0.26	14.8 ± 0.18	15.3 ± 0.35
Body composition, % :				
Water	69.7 ± 1.3	71.7 ± 0.6	66.9 ± 1.2	65.6 ± 1.3
Protein	16.5 ± 0.7	16.7 ± 0.4	17.4 ± 0.5	17.7 ± 0.5
Lipids	10.0 ± 0.9	6.2 ± 0.4	10.3 ± 0.9	12.1 ± 0.9
Ash	3.0 ± 0.3	3.4 ± 0.1	3.5 ± 0.3	3.5 ± 0.2
Vitamin B_6,[2] μg per:				
Gram liver, wet wt	9.6 ± 0.2	3.9 ± 0.3	3.8 ± 0.4	2.8 ± 0.2
Gram kidney, wet wt	4.4 ± 0.5	1.5 ± 0.1	1.8 ± 0.2	1.7 ± 0.1
100 ml of blood	20.6 ± 3.4	1.6 ± 0.2	1.7 ± 0.2	2.0 ± 0.6

[1] Values include SE of mean.
[2] Expressed as pyridoxine.

consumption. The tail length measurements and body composition data indicate that insulin administration resulted in an increase in bone growth and all body fractions. Insulin treatment had a greater effect in increasing carcass lipids than on other body components. Since insulin treatment did not increase vitamin B_6 levels in the liver, kidney and blood of the vitamin B_6-deficient rats, it is not likely that the action of insulin was the result of conservation of vitamin B_6.

Table 2 shows the effect of vitamin B_6 deficiency on the fatty acid composition of the epididymal fat pads of rats pair-fed the basal diet with and without vitamin B_6 for 3 weeks. This table also shows the effect of vitamin B_6 deficiency with and without insulin treatment (one unit/rat/day) on the fatty acid composition of livers and epididymal fat pads of rats fed ad libitum for 3 weeks a diet contaning 4% trimyristin instead of 4% corn oil to reduce the effect of dietary fat composition on the fatty acid composition of the tissue studied. Values for lauric, myristoleic, palmitic and oleic acids have not been shown because they were not affected. In the vitamin B_6-deficient rats, palmitoleic acid was significantly decreased, whereas stearic and linoleic acids were significantly increased in both liver and epididymal fat pad lipid. Insulin partially reversed these changes. The increased linoleic acid in the deficient rats did not appear to be due to reduced conversion to arachidonic acid since the liver arachidonic acid levels were not affected by the deficiency. It appears that the fatty acid changes observed in vitamin B_6 deficiency are not directly due to a lack of the vitamin, but may, at least in part, be the consequence of reduced insulin. Decreased palmitoleic acid and increased stearic and linoleic acids were also observed in the epididymal fat pads of pair-fed vitamin B_6-deficient and control rats. This suggests that the fatty acid changes observed in vitamin B_6-deficient rats are not the consequence of reduced food intake.

Effect of vitamin B_6 deficiency on blood glucose and glucose tolerance. Tail blood was collected from several groups of vitamin B_6-deficient and control rats for glucose determinations. The results of these

TABLE 2

Effect of vitamin B_6 deficiency and insulin treatment on epididymal fat pad and liver fatty acids

Dietary fat	Treatment [2]	No. of rats	Organ	Myristic [1]	Palmitoleic [1]	Stearic [1]	Linoleic [1]	Arachidonic [1]
4% Corn oil	With vitamin B_6 [3]	10	fat pad	2.1 ± 0.1 [4]	10.6 ± 0.7	1.7 ± 0.1	11.8 ± 1.0	trace
1% Cod liver oil	Without vitamin B_6	10	fat pad	2.1 ± 0.1	6.2 ± 1.8	3.0 ± 0.5	17.8 ± 1.2	trace
4% Trimyristin	With vitamin B_6	6	fat pad	17.3 ± 0.8	16.3 ± 0.9	1.2 ± 0.06	0.2 ± 0.07	trace
1% Cod liver oil	With vitamin B_6 and insulin	6	fat pad	16.2 ± 1.3	17.7 ± 0.7	0.6 ± 0.06	0.2 ± 0.02	trace
	Without vitamin B_6	6	fat pad	17.3 ± 0.3	11.2 ± 0.6	2.1 ± 0.1	0.6 ± 0.14	trace
	Without vitamin B_6 and with insulin	6	fat pad	16.3 ± 0.8	14.0 ± 0.1	1.0 ± 0.2	0.5 ± 0.1	trace
	With vitamin B_6	6	liver	3.2 ± 0.05	8.8 ± 0.6	9.6 ± 1.1	0.4 ± 0.1	4.6 ± 1.3
	With vitamin B_6 and insulin	6	liver	2.2 ± 0.03	10.0 ± 2.0	3.4 ± 0.7	0.2 ± 0.1	1.4 ± 0.4
	Without vitamin B_6	6	liver	1.7 ± 0.6	4.5 ± 0.6	17.5 ± 2.7	2.3 ± 0.7	5.2 ± 2.0
	Without vitamin B_6 and with insulin	6	liver	2.9 ± 0.6	7.0 ± 1.1	9.2 ± 1.8	1.5 ± 1.0	5.0 ± 1.7

[1] Figures represent % of individual fatty acids in total fatty acids extracted from tissues studied.
[2] When used during the last 2 weeks of the 3 week experimental period, 1 unit of Protamine-Zn-Insulin was injected intraperitoneally per day.
[3] Pair-fed.
[4] Values include SE of mean.

studies shown in table 3 indicate that vitamin E_6-deficient rats are hypoglycemic when compared with control rats in both fasted and non-fasted states.

Two groups of 6 weanling rats were fed the basal diet with and without vitamin B_6 for 3.5 weeks. After an 18-hour fast, the animals were anesthetized with pentobarbital sodium[4] (5 mg/100 g body weight) and injected in a tail vein with 100 mg sterile glucose solution/100 g body weight. Subsequently over a period of one-half hour, 0.1 ml blood samples were drawn from the tip of the tail for blood glucose determinations. The disappearance of glucose during this period followed straight lines when the logarithms of the glucose concentrations were plotted against time. The logarithmic difference of the glucose concentrations in milligrams per 100 ml of blood between 5 and 25 minutes after the injection, was for the control group 0.297 ± 0.02, and was 0.125 ± 0.07 for the vitamin B_6-deficient group. The difference in slopes is statistically significant ($P < 0.001$) and indicates that the disappearance of glucose in the vitamin B_6-deficient group was considerably slower than in the control group.

Effect of vitamin B_6 deficiency on pancreas and serum insulin-like activity. Insulin-like activity was determined in both the sera and the pancreata of vitamin B_6-deficient and control rats. Blood was obtained from the inferior vena cava under light ether anesthesia. After standing at room temperature for one hour, it was centrifuged and the serum separated. Sera of 3 to 5 rats had to be pooled for one estimation. The glucose content was determined in each sample. Insulin was extracted from the pancreata according to Scott and Fisher (18) and dissolved in incubation medium for assay as described above.

The results of these studies are presented in table 4. In both of the experi-

[4] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.

TABLE 3

Glucose in blood of vitamin B_6-deficient rats

Diet	Rats/ group	Weeks fed diet	Sample	Glucose		P value
				Control	Without vitamin B_6	
				mg/100 ml	*mg/100 ml*	
Basal	6	5	Blood, non-fasted	123 ± 2.0 [1]	83 ± 4.5	0.001
One week lab chow, then basal	10	4	Serum, non-fasted	320 ± 17.4	250 ± 4.5	0.001
Basal	12	4	Blood, fasted 18 hr	85 ± 2.8	67 ± 3.9	0.01

[1] Values include SE of mean.

TABLE 4

Insulin-like activity in serum and pancreas of vitamin B_6-deficient rats

Diet	Method	Food removed prior to killing	Weeks diet	No. samples/ group	Insulin-like activity		P value
					With vitamin B_6	Without vitamin B_6	
					$\mu U/ml$ serum		
Basal	Renold (16)	4 hr	4	10	164 ± 57 [1]	47 ± 12	< 0.05
Basal	Renold (16)	not removed	5	14	291 ± 44	248 ± 43	NS [2]
Basal	Ball et al. (17)	3 hr	4	6	232 ± 75	32 ± 16	< 0.05
Trimyristin	Ball et al. (17)	4 hr	6	7	225 ± 37	44 ± 23	< 0.01
High fat	Ball et al. (17)	3 hr	4	6	95 ± 10	69 ± 17	NS
					$\mu U/g$ pancreas		
Basal	Renold (16)	4 hr	4	8	1.45 ± 0.31	0.65 ± 0.19	< 0.05
Basal	Ball et al. (17)	3 hr	5	6	1.34 ± 0.16	0.52 ± 0.13	< 0.02

[1] Values include SE of mean.
[2] Not significant.

ments in which the basal diets were fed, pancreatic insulin-like activity was significantly reduced in the vitamin B_6-deficient rats. The circulating insulin-like activity was significantly reduced in deficient rats fed this diet and the diet containing trimyristin as a fat source. In the one experiment, in which the food intake was not limited, the mean value was not significantly lower in the deficient group. When the rats received a high fat diet, both the control and deficient rats showed low serum values and the difference was not statistically significant although the mean value was lower in the vitamin B_6-deficient group.

Effect of vitamin B_6 deficiency on epididymal fat pad activity. Since one of the major sites of insulin action is on adipose tissue, the in vitro effect of insulin on this tissue was studied. Two groups of eight weanling rats were pair-fed the basal diet with and without vitamin B_6 for 5 weeks. From each animal 4 pieces of 100 to 150 mg of their epididymal fat pads were incubated separately at 37°C in Warburg flasks in an atmosphere of 95% O_2-5% CO_2 at pH 7.4 in Krebs-Ringer carbonate buffer containing 0.5 mg gelatin, 4 mg (0.08 μc) of uniformly labeled glucose and 200 μunits of glucagon-free insulin/ml. One-half of the flasks also contained 1 μg of pyridoxal and 1 μg of pyridoxal-5-phosphate/ml of medium. Before the insulin was added from the sidearms, net gas exchange was measured for one hour and was found to be slightly negative, about -3 to -4 μl/100 mg fat pad. However, no significant differences were observed in net gas exchange of the fat pads of the control and vitamin B_6-deficient animals unless insulin was added to the medium. After the addition of insulin, net gas exchange was measured every one-half for 2.5 hours during which period it was linear for both deficient and control animals. At the end of the incubation period, the fat pads were washed with saline and blotted 6 times to remove traces of medium. They were then homogenized with a 2:1 chloroform, methanol mixture and filtered. From each protein-free extract aliquots were taken for the measurement of lipid ester bonds (19) and for the counting of radioactivity in a gas flow counter.

The results shown in table 5 indicate that net gas exchange and incorporation of C^{14} into fat was significantly greater in the fat pads of vitamin B_6-deficient rats than those of the controls. There was a high degree of correlation between net gas exchange and incorporation of C^{14} into fat ($r = +0.86$).

The addition of pyridoxal and pyridoxal-5-phosphate to the incubation medium had no statistically significant effect on net gas exchange or incorporation of C^{14} into fat, although slightly high values were found when they were added.

In a second study, 3 groups of 8 weanling rats, 2 groups vitamin B_6-deficient, were fed the basal diet ad libitum for 5 weeks. The rats of one of the deficient groups were injected with 4 mg of pyridoxal 24 hours prior to killing. The food intake of this group was restricted during the last day to the level consumed by the untreated vitamin B_6-deficient group. The epididymal fat pads of the rats were treated as described above, except that neither labeled glucose, pyridoxal, nor pyridoxal-5-phosphate were added to any of the flasks.

TABLE 5

Effect of vitamin B_6 deficiency on epididymal fat pad activity

	Pyridoxal and pyridoxal-5-PO_4 in medium	Diet with vitamin B_6	Diet without vitamin B_6	*P* value
μl net gas exchange/				
100 mg fat pad/hr	—	28 ± 5 [1]	69 ± 11	< 0.01
	+	34 ± 7	78 ± 12	< 0.01
Count/min/μEq. of ester	—	1.69 ± 0.26	2.51 ± 0.32	NS [2]
	+	1.81 ± 0.22	2.91 ± 0.40	< 0.05

[1] Each value represents the mean ± SE of mean of observations made on 8 pair-fed animals.
[2] Not significant.

TABLE 6

Effect of in vivo pyridoxal administration on epididymal fat pad activity in vitamin B_6-deficient rats

	Diet		
	With vitamin B_6	Without vitamin B_6	Without vitamin B_6 [1]
Net gas exchange, μl/100 mg fat pad/hr	13.0 ± 2.2 [2]	32.0 ± 3.9	33.0 ± 4.8
μl/100 μg N/hr	7.7 ± 2.0	13.3 ± 1.3	17.2 ± 2.7

[1] Each rat injected with 4 mg of pyridoxal 24 hours prior to killing.
[2] Each value represents the mean ± SE of mean of observations made on 8 animals.

The results of this study are shown in table 6. They indicate that when the fat pads of deficient rats given a single large dose of pyridoxal 24 hours prior to killing were incubated with insulin and glucose, net gas exchange was not significantly different than that observed when the fat pads of untreated vitamin B_6-deficient rats were similarly incubated. Similar conclusions are drawn whether the results are expressed on a wet weight or nitrogen basis.

DISCUSSION

Evidence exists which suggests the possibility that insulin is deficient in vitamin B_6-deficient rats. Tezuka and Makino (20) and Koide (21) reported mild hyperglycemia and glucosuria in this deficiency. The animals studied here were hypoglycemic, as has been reported by Beaton and Goodwin (5). Urinary glucose excretion was negligible, and urinary ketone excretion was normal. No apparent explanation for the differences observed in these studies and those of the Japanese is available. However, in agreement with Koide (21) the rate of removal of glucose from the blood after a glucose load was abnormally slow in vitamin B_6-deficient rats. Other evidence has been provded that changes observed in vitamin B_6-deficient rats may be partially reversed by insulin administration. However, these animals are not typically diabetic.

Beaton et al. (7) showed that the administration of insulin to vitamin B_6-deficient rats improved their growth. This has been confirmed and extended. Carcass analyses indicated that the main abnormality in the body composition of weanling rats fed vitamin B_6-deficient diets for 4 weeks is a decrease in the percentage of fat and that this can be prevented by insulin administration.

The composition of the tissue fatty acids was also altered by vitamin B_6 deficiency. In the experiments reported here, and others not reported, these changes did not occur in pair-fed control animals. In deficient rats receiving either corn oil or trimyristin as their main fat source, tissue levels of palmitoleic acid decreased and stearic and linoleic acids increased. Palmitoleic acid was not present in the dietary fats in appreciable amounts and the lower content of this fatty acid in the tissues presumably reflected a decrease in its rate of synthesis. Linoleic acid is not synthesized. Thus, the relatively large quantities in the deficient animals may reflect a lower rate of destruction. The administration of insulin to vitamin B_6-deficient rats resulted in a reversion of the fatty acid content of the tissues toward normal, both in amount and composition. In view of our inability to control independently the rates of synthesis, deposition and mobilization of fatty acids, the exact role of insulin is unclear from these studies.

An obvious possibility to explain the insulin effect would be the conservation of vitamin B_6 by the body. Liver, kidney and blood concentrations of vitamin B_6 in the animals were not increased by the administration of insulin. However, it is possible that tissues not studied or parts of tissues conserve vitamin B_6 under the influence of insulin.

The evidence that insulin reverses or prevents many of the changes accompanying vitamin B_6 deficiency is associated with the hypothesis that there is a decreased availability of insulin. It is realized that the methods used are measures of insulin-like activity and until the specificity of the methods is better defined, the results must be interpreted with caution. Nevertheless, the data show a decrease in insulin-like ac-

tivity in both the sera and pancreata of the vitamin B_6-deficient rats. It is not necessarily clear that a decreased availability of insulin is one of the first manifestations of vitamin B_6 deficiency. However, within 24 hours after young rats are fed the vitamin B_6-deficient diet, there is a considerable decrease in food intake which can be prevented by insulin administration.

It is well known that appetite can be stimulated by insulin administration. However, we are unaware of any studies in which the anorexia accompanying a deficiency of an essential nutrient other than vitamin B_6 has been prevented by insulin administration. In unreported studies from this laboratory the administration of insulin to thiamine-deficient rats did not result in an increase in growth or food intake. Unlike vitamin B_6-deficient rats, thiamine deficient rats did not show a greater sensitivity to insulin than their controls. In a study preliminary to the work reported here, daily injections of zero to 10 units of insulin/day were given to weanling vitamin B_6-deficient and control rats. None of the deficient rats survived more than 6 units/day. The controls tolerated 10 units/day when food was provided ad libitum. Increased sensitivity of vitamin B_6-deficient rats to insulin has also been mentioned by Beaton et al. (7). In the present study it was also observed that the fat pads of deficient rats exhibit much greater responses than those of control rats to the same level of insulin in vitro as shown in tables 5 and 6. Significant differences in net gas exchange between fat pads of vitamin B_6-deficient and control rats have not been observed in the absence of insulin. Thus it appears that the RQ of fat pads from vitamin B_6-deficient rats in the presence of a glucose substrate is greater than that of control animals only when insulin is added to the medium. The data presented from the in vitro studies indicate that in the presence of equal amounts of added insulin the fat pads of vitamin B_6-deficient rats synthesize fat from glucose more rapidly than the fat pads of control animals.

One of the first signs of vitamin B_6 deficiency is decreased food intake and growth failure. Paired feeding experiments show clearly that there is inefficient energy utilization in the deficient animals (4). They gain less rapidly with the same caloric intake. The work of Carter and Phizackerley (3) and unreported studies from this laboratory indicate that this is not the result of faulty intestinal absorption or loss of calories through increased urinary excretion. It is difficult to assess how many of the metabolic changes of vitamin B_6 deficiency are the result of inanition. Paired feeding experiments are of value in minimizing the effects of inanition, but the value of the paired feeding method is limited by the necessity to restrict the food intake of one of the groups. In the present studies it was impossible to pair-feed vitamin B_6-deficient rats receiving insulin with untreated deficient animals because they could not be kept alive on a restricted feeding program. The administration of insulin to vitamin B_6-deficient rats resulted in an immediate increase in food consumption with a concomitant increase in body size. However, it should not be concluded that the metabolic changes of vitamin B_6 deficiency reported here are the result of inanition. The decrease in palmitoleic acid and the increase in stearic and linoleic acids reported in the fat pad and the liver lipid of vitamin B_6-deficient rats were not observed in the epididymal fat pads of pair-fed control animals (table 2). The increased activity of the epididymal fat pads of vitamin B_6-deficient rats in the presence of insulin was also observed in both pair-fed (table 5) and ad libitum-fed animals (table 6). Beaton and Goodwin (5) have reported that vitamin B_6-deficient rats showed lower fasting liver glycogen and lactic acid dehydrogenase activity and lower fasting blood sugar, pyruvic acid and lactic acid levels than their pair-fed controls. Beaton (22) has also reported that insulin administration has a slightly more pronounced effect on blood sugar levels in vitamin B_6-deficient rats than in pair-fed controls.

The simultaneous occurrence of hypoglycemia, decreased availability of insulin and increased sensitivity to insulin suggest the possibility of a pituitary or adrenal derangement in vitamin B_6-deficient rats. It is well known that hypophysetcomy or adrenalectomy of the diabetic aniaml leads to amelioration of hyperglycemia, and it has

been reported repeatedly that hypophysectomized animals are more sensitive to insulin. Furthermore, Reit-Correa et al. (23) have shown that cortisone injected into the rat reduces the sensitivity of the epididymal fat pad to small doses of insulin. In view of the changes observed it is possible that changes in insulin availability may simply be an adaptation to a state in which less insulin is required.

That food intake in the rat may be modified within 24 hours of the time it is fed a vitamin B_6-deficient diet, and before large changes in total body concentrations of the vitamin occur, indicates that there may be specific centers which are very sensitive to changes in vitamin concentration. These might include the endocrine glands or hypothalamic centers, such as those known to be intimately associated with the control of food intake.

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Tissue Distribution of α-Aminoisobutyric Acid and Nitrogen Metabolism in the Rat

II. EFFECTS OF ENVIRONMENTAL TEMPERATURE AND DIETARY IMBALANCE[1]

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ABSTRACT Exposure to cold (7°C) resulted in rats increasing their food intake, independently of the nature of their diets. In spite of this, the nitrogen retention of these animals was lower than that of their respective controls maintained at room temperature (24°). The addition of toxic levels of amino acids to the diet of animals maintained at 24° greatly decreased the ratio of weight gain per gram of retained nitrogen. On the other hand, animals fed similar diets but exposed to the low environmental temperature were able to gain more weight in terms of the retained nitrogen. Since this increase in weight cannot be attributed to an increase in net protein synthesis over that of their controls, accumulation of other products such as lipids or water may be responsible. Exposure to cold, as well as the supplementation of the diets with toxic amounts of tryptophan or an isoleucine-free amino acid mixture, stimulated the urinary excretion of injected α-aminoisobutyric acid, with more rapid depletion of this unmetabolizable amino acid from the tissues.

It is generally well-recognized that incomplete or imbalanced diets, inadequate to support optimal growth, result in decreased food intake. This depression of food consumption cannot be attributed to low palatability of the diets and is probably related to some systemic effect which causes food rejection (1, 2). Attempts to overcome this growth retardation by force-feeding these animals have been unsuccessful and even detrimental (3, 4).

However a recent report indicated that exposure to cold was able to overcome, in certain instances, the growth retardation caused by amino acid-imbalanced diets (5). The mechanism involved in this behavior has not been advanced. In no instance was this response to cold exposure nearly as adequate as that obtained by feeding isonitrogenous amounts of a balanced supplement.

In any case, it appears reasonable to expect that the beneficial effect of cold on the growth of rats fed imbalanced rations should be associated with an increased retention of nitrogen or its more efficient utilization. The following experiments were initiated to investigate these possibilities: the feeding of diets with imbalanced amino acid mixtures, amino acid deficiencies and marked excesses. In addition to effect on growth and nitrogen balance, the distribution as well as the excretion, of the unmetabolizable amino acid α-aminoisobutyric (AIB) was investigated. Recent experiments from our laboratory indicate that the nature of the diet, especially the protein level, greatly influenced the retention, distribution and pattern of urinary excretion of tracer doses of injected AIB (6).

EXPERIMENTAL

Young, growing male Holtzman rats of 100 to 110 g in body weight were used in these experiments. They were divided into 2 groups; one maintained at room temperature (24°C) and another in the cold (7°). The animals were housed in stainless steel metabolic cages, food intake was measured daily, and urine and feces were collected daily. The basal 24 and 9% casein diets were made up as previously described (7). The 6% fibrin diet was similar, with this protein replacing casein. At these low levels of dietary fibrin, isoleucine has been shown to be one of the most limiting amino acids on the growth of the rat (8). An isoleucine-free amino acid mixture, sim-

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TABLE 1

Changes in body weight and nitrogen balance of animals fed different imbalanced diets while maintained at room temperature (24°C) and in a cold environment (7°C)

Group	Environment	Body wt increase	Food intake	Nitrogen retention	RNER [1] increase body wt/g N retention	Protein efficiency ratio
		g/day	*g/day*	*mg/day*		
24% Casein	room temperature	7.0 ± 0.3 [2]	16.6	386 ± 10	18.1 ± 0.4	1.88 ± 0.12
	cold	4.1 ± 0.2	21.9	232 ± 14	17.7 ± 1.3	0.85 ± 0.17
24% Casein + 3% tryptophan	room temperature	3.8 ± 0.3	15.1	340 ± 21	11.2 ± 1.3	1.15 ± 0.12
	cold	2.5 ± 0.6	18.3	122 ± 25	20.4 ± 0.6	0.60 ± 0.1
9% Casein	room temperature	5.4 ± 0.4	18.8	198 ± 12	27.3 ± 1.1	3.70 ± 0.12
	cold	3.8 ± 0.2	21.5	173 ± 9	22.0 ± 1.1	2.26 ± 0.11
9% Casein + 3% leucine	room temperature	0.8 ± 0.1	13.0	101 ± 7	7.9 ± 1.2	0.78 ± 0.43
	cold	1.7 ± 0.2	18.3	69 ± 2	24.7 ± 2.8	1.21 ± 0.11
6% Fibrin	room temperature	3.4 ± 0.2	16.5	130 ± 5	26.2 ± 1.2	3.40 ± 0.14
	cold	3.1 ± 0.2	21.4	141 ± 5	22.0 ± 2.0	2.40 ± 0.18
6% Fibrin + 4.5% amino acid mixture	room temperature	2.3 ± 0.2	12.7	107 ± 9	21.5 ± 2.0	1.94 ± 0.16
	cold	2.5 ± 0.6	19.9	96 ± 13	26.0 ± 4.9	1.35 ± 0.35

[1] Indicates retained nitrogen efficiency ratio.
[2] SE.

ilar to the one used by Kumpta and Harper (9) except for the omission of tryptophan, was used. It contained DL-methionine, 9%; DL-phenylalanine, 13.5%; L-leucine, 9%; DL-valine, 15.5%; L-lysine·HCl, 13.5%; DL-threonine, 4.5%; L-arginine, 4.5%; L-glutamic acid, 22%; and L-histidine, 9%. This amino acid imbalancing mixture, when added to the diet, supplied an extra 4.5% of amino acids above those contributed by fibrin.

All the animals were injected subcutaneously with 1 μc of AIB-1-C^{14}/100 g of body weight (specific activity 6.05 mc/ml), 3 days after starting to be fed the experimental diets and killed 3 weeks following the isotope injection. Serum, tissue and urine samples were treated as previously described for the purpose of determining residual radioactivity (6). Total nitrogen in the diet and urine was determined colorimetrically after digestion using the Nessler reagent.

The protein efficiency ratio (PER) represents the gain in body weight in grams resulting from the intake of 1 g of protein. A retained nitrogen efficiency ratio (RNER) was also calculated, which would reflect the gain in body weight in grams which accompanies the retention of 1 g of nitrogen.

RESULTS

The changes in body weight, the food intake as well as the calculated nitrogen balance values recorded during the course of these experiments, are summarized in table 1. In all instances exposure to cold increased the food consumption. The animals fed the 24% casein diet grew less in the cold in spite of their increased food intake. The decreased nitrogen retention of these animals is also reflected in their decreased growth. The efficiency with which this retained nitrogen was utilized for growth was similar in both groups. In contrast, the PER was decreased in the group exposed to cold.

Addition of toxic amounts of tryptophan to the diet retarded growth of both the controls and cold-exposed animals. Cold exposure again produced a decrease in the nitrogen retention. However, when measured in terms of utilization of retained

Fig. 1 Urinary excretion of C^{14} following the injection of a single dose of α-aminoisobutyric acid-1-C^{14} to rats. Animals were fed diets differing in protein quality and quantity, and supplemented with toxic levels of a single amino acid or an amino acid imbalancing mixture free of isoleucine. In each experiment one group was maintained at 24°C and one at 7°. Values are the average of 5 animals, expressed as percentage of the injected dose excreted.

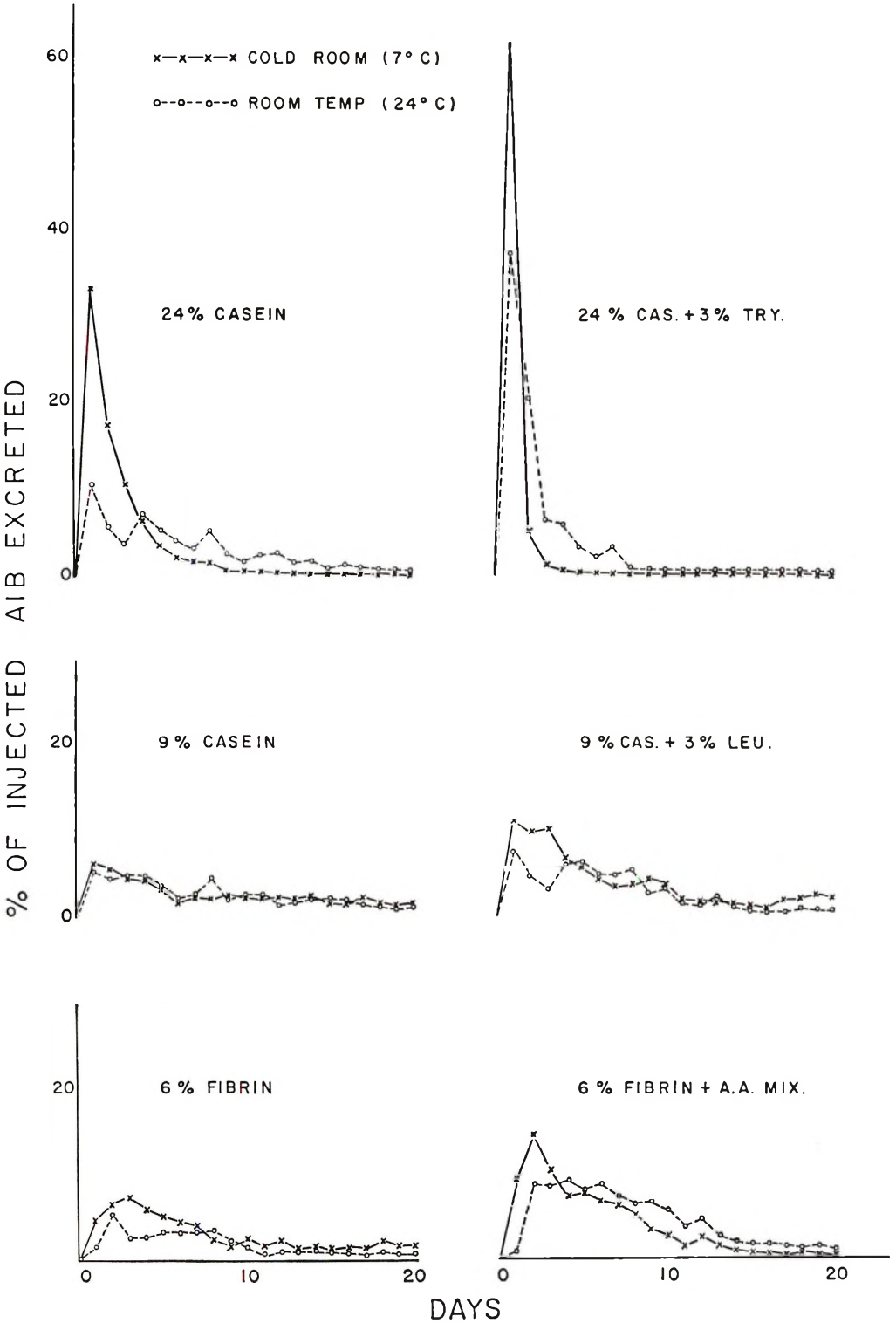
x—x—x—x COLD ROOM (7° C)
o--o--o--o ROOM TEMP (24° C)
% OF INJECTED AIB EXCRETED
60
40
20
0
24% CASEIN
24 % CAS. + 3% TRY.
20
0
9 % CASEIN
9 % CAS. + 3 % LEU.
20
6 % FIBRIN
6 % FIBRIN + A.A. MIX.
0
10
20
DAYS

Figure 1

nitrogen (RNER) the animals exposed to the cold appear to have utilized it much more efficiently. When dietary casein was supplied at the 9% level, exposure to cold again retarded growth. Retention of nitrogen was also reduced but not significantly.

The addition of 3% leucine to the 9% casein diet produced a marked inhibition of growth when fed to animals maintained at 24°. However, animals maintained at 7° performed better as judged by growth, RNER, and PER ratios. On the other hand, animals fed the 6% fibrin diet were not affected in the above parameters when exposed to the colder environment. The addition to this diet of an imbalancing mixture of amino acids lacking isoleucine further stunted the growth of these animals. However, as in the previous experiment the growth rate of these animals was not affected by temperature.

The PER calculated throughout these experiments tended to be greater at room temperature, with the exception of the animals receiving toxic amounts of leucine mixed in a 9% casein diet where the values were reversed.

The urinary excretion patterns of the radioactive AIB injected at the beginning of the experiment, are shown in figure 1. Each point of the curve represents the average urinary radioactivity of that group, for a 24-hour period, expressed as a percentage of the injected dose. Those animals consuming the higher levels of casein excreted initially the greatest amount of the unmetabolizable amino acid. The addition of 3% tryptophan to the diet of the animals resulted in a marked increase in excretion of AIB in both the group maintained at 7° as well as those kept at 24°. However, no differences were observed when animals were fed the 9% casein diet unsupplemented with excess tryptophan. In animals fed the 6% fibrin diet, AIB excretion increased when an imbalancing amino acid mixture was fed.

The addition of toxic amounts of amino acids, or of imbalancing amino acid added to the diets of the experimental animals resulted in a marked increase in the urinary excretion of tracer doses of administered AIB. Simple exposure to cold had similar effects in all groups except in those animals fed the 9% casein diet. All the animals in the experiment were killed at the end of 3 weeks and tissues were analyzed for radioactivity. The values obtained are recorded in table 2.

Exposure to cold as well as the level and nature of the protein in the diet greatly influenced the amount of AIB retained by the animal. As expected, the amounts excreted in the urine maintained a reverse

TABLE 2

Residual tissue radioactivity 21 days following the administration of α-aminoisobutyric acid-1-C^{14} to rats fed different imbalanced diets while maintained in the cold room and at room temperature

Diet	Environment	Serum radioactivity	Tissue radioactivity		
			Liver	Kidney	Skeletal muscle
		count/min/ml		*count/min/g*	
24% Casein	room temperature	128	270	2140	530
	cold	0	10	30	10
24% Casein + 3% tryptophan	room temperature	0	10	30	0
	cold	0	0	20	0
9% Casein	room temperature	320	330	5130	1160
	cold	190	380	3570	750
9% Casein + 3% leucine	room temperature	592	1720	12180	1080
	cold	72	260	2340	400
6% Fibrin	room temperature	608	670	5950	1660
	cold	304	600	5660	790
6% Fibrin + 4.5% amino acid mixture	room temperature	72	110	2340	250
	cold	0	20	220	20

relationship with the amounts retained in the tissues. On the other hand, the distribution of radioactivity among the various tissues studied for all the animals in the test follows a similar pattern. Calculation of the distribution ratio, tissue radioactivity-to-serum radioactivity (where the serum value is used as a standard of comparison) shows this to be true. The large kidney uptake observed in all instances, reflects the physiological concentrative capacity of this organ, prior to AIB excretion in the urine.

DISCUSSION

In agreement with the observations of Klain and co-workers (5), exposure to low temperature increased the food consumption of rats in all of our experimental groups. However, in spite of increased food intake the total nitrogen retention of these animals was lower than that of their respective controls maintained at 24°. However, the addition of toxic amounts of leucine to a 9% casein diet resulted in better growth in the animals maintained in the cold, compared with controls maintained at 24°. However, although these animals ate more food, their nitrogen retention was considerably less.

The animals fed the fibrin diet, as well as those fed the same diet plus an imbalancing amino acid mixture, grew equally as well regardless of the environmental temperature. This contrasts with what occurs during the feeding of casein. Under these circumstances cold exposure retarded growth, and the addition of toxic amounts of tryptophan to the diet produced a further depression of weight gain.

The addition of toxic levels of amino acids to the diet of animals maintained at 24° greatly decreased the efficiency of utilization of the retained nitrogen with respect to growth. On the other hand, animals fed identical diets but exposed to the low environmental temperature were able to gain more weight in terms of the retained nitrogen. It cannot be ascertained at this time whether this increase in weight is due to protein synthesis or to the accumulation of other products such as lipids or water. Based on our present knowledge concerning protein synthesis, it is difficult to explain how a smaller amount of retained nitrogen can generate a larger amount of organized tissue. But it is permissible to think that the increased caloric intake of the cold-exposed animals could lead to a deposition of other nonprotein metabolic products. Body composition studies are needed to ascertain the biochemical nature of the weight changes observed.

Exposure to cold, as well as the supplementation of a balanced diet with a toxic amount of tryptophan stimulated the urinary excretion of injected AIB. The combination of cold and amino acid toxicity exhibited additive effects. At low levels of dietary protein the effects induced by amino acid toxicity or imbalance were similar in nature but much less pronounced. Exposure to cold in most instances tended to deplete more rapidly the stores of AIB from the tissues. These observations are a possible reflection of the increased nitrogen loss which takes place during exposure to cold resulting in a decrease in nitrogen retention in spite of the increased intake. The nature of the weight gain of the animal exposed to cold and consuming imbalanced diets with low levels of protein requires further investigation. Long-term studies are being performed to evaluate the effects and implications of this stimulation in growth.

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Amino Acid Composition of Rye Flour and the Influence of Amino Acid Supplementation of Rye Flour and Bread on Growth, Nitrogen Efficiency Ratio and Liver Fat in the Growing Rat[1]

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ABSTRACT The amino acid compositions of 4 rye flours were similar except in the lysine content. Compared with the FAO reference pattern, rye protein appears to be low in methionine and cystine, tryptophan, lysine and isoleucine. For the growing rat the calculated deficiencies appear to be lysine, methionine and cystine, isoleucine or threonine, and tryptophan. In rat feeding experiments lysine was the first and threonine the second most limiting amino acid. No single amino acid or pair of acids increased the protein value of rye flour supplemented with lysine and threonine. This required a group of amino acids in which isoleucine appeared to be indispensable. A rye diet containing 8 times the optimal amount of L-lysine·HCl was still superior to a nonsupplemented rye diet and the addition of DL-threonine or L-isoleucine alone to a nonsupplemented rye diet had little effect on growth, nitrogen efficiency ratio and liver fat content. However, when essential amino acids other than threonine were added to rye diets fortified with lysine, a lowering of the protein value followed. This could be counteracted by threonine supplementation. Rye flour had a protein value superior to that of wheat flour but rye crisp bread was inferior to soft wheat bread.

Rye, *Secale cereale,* is a cereal that does not attract the same world-wide interest as for instance wheat, maize or rice. The total world production of rye is also comparatively small, about 37 million metric tons as compared with about 244, 224 and 240 million metric tons for wheat, maize and rice (1). However, in U.S.S.R., Poland, Germany, Sweden and some other countries, rye still occupies a position among the cereals significant enough to warrant attention from a nutritional point of view.

An investigation of the amino acid composition of rye flour and the effect of fortification with free amino acids on the protein value of rye flour and rye bread was a logical extension of our previous work on wheat and wheat products (2–4), teff (5) and leguminose products (6). The amino acid analyses were carried out using chemical and microbiological methods and the results compared with the amino acid requirements of humans and of the growing rat. The protein value of rye flour and bread before and after amino acid supplementation was estimated by a rat growth method.

EXPERIMENTAL

Conditions of the animal experiments. Young male albino rats of the Sprague-Dawley strain with an initial weight of about 45 g were used in all the feeding experiments. They were arranged in such a way as to give experimental groups of approximately the same mean weight and weight distribution. The animals were housed individually in wire-bottom cages in an air conditioned room kept at a temperature of 25°C and a relative humidity of 50 to 55%.

Food and water was fed ad libitum. Before being fed the various experimental diets, a basal rye diet was given for a few days to accustom the weanling rats to the new living and feeding conditions. This may also have served to standardize the rats with respect to their protein stores. Weighing took place twice a week and food consumption was measured individually at the end of the experimental period. Losses of feed through spillage were gen-

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erally insignificant due to the type of feeder used. At the end of the experimental period each animal was stunned by a blow on the head, decapitated and bled, whereafter the liver was removed immediately.

The diets.[2] All diets contained (in per cent): salt mixture, 3; soybean oil, 5; cod liver oil, 0.5; choline chloride, 0.15; and 147 mg/kg of a vitamin mixture. In most cases, 91% rye flour or ground rye bread supplied all the protein and carbohydrate of the diets. When diets had to be adjusted with respect to the protein content, this was done by the inclusion of wheat starch; in such diets, flour, bread or casein plus wheat starch made up 91% of the diet weights.

The salt mixture was the same as that of Hegsted et al. (7) and the vitamin mixture was identical to that of Harper et al. (8).

Four different mixtures of rye flour were studied. Their origin, water and nitrogen content is shown in table 1. The extraction rate of all the rye flours was approximately 98%. As indicated in the table, the 4 types have been designated "rye A to D." Besides rations made from the 4 rye flour mixtures, diets based on Swedish rye crisp bread, wheat crisp bread, soft white bread and wheat flour were also fed in order to estimate the loss of protein value due to baking and to compare the protein values of wheat and rye flour and bread. All breads were commercial products of wide acceptance. The soft white bread was sliced, dried at 20°C for 3 days and ground. The wheat flour was the same as that used in previous feeding experiments (3).

TABLE 1

Origin of and the water and nitrogen content of the types of rye flour studied

Rye	Water content	Nitrogen [1] content	Russian rye	Swedish rye	Argentinian rye
	%	%	%	%	%
A	12.8	2.03	100	—	—
B	13.9	1.75	50	50	—
C	13.2	1.47	20	80	—
D	12.0	1.79	70	—	30

[1] Expressed as percentage of the fresh weight of the flour.

The nitrogen content of the various experimental diets is given in the headings of the tables of results.

Analytical procedures. Dry weight determinations were carried out by heating the samples at 104 to 105° for 24 hours, cooling in a desiccator over silica gel and weighing. Nitrogen content was determined by the modified Kjeldahl procedure of Perrin (9). Liver fat content was estimated on dried and ground samples by the procedure recommended by the AOAC (10).

The amino acid composition of the 4 different rye flours, with the exception of tryptophan and cystine, was determined by chromatography on ion exchange resins according to Spackman et al. (11). The samples were defatted and hydrolyzed in sealed, evacuated ampules in 6 N HCl (4 ml to 0.2-g sample at 105 to 110° for about 40 hours).

Tryptophan was estimated microbiologically using a turbidimetric tube assay with *Leuconostoc mesenteroides* ATCC 8042. The assay medium was basically the same as that used by Bolinder and Larsen (12) for the determination of niacin but with the omission of tryptophan and the addition of 1 mg niacin/liter single strength medium. The assay tubes were incubated at 37° for 20 hours and the measurement of turbidity was made at 640 mμ.

Various methods for the liberation of the tryptophan were tried. In one series of experiments, flour samples were defatted and mixed with powdered barium hydroxide and water as recommended by Hepburn et al. (13). The mixtures were heated at 122° in sealed ampules for 10, 15, 20, and 40 hours. Both Pyrex and quartz ampules were used. In other series, enzymatic liberation with pepsin, trypsin and erepsin according to Barton-Wright (14) or with a broad-spectrum protease[3] was tried. In the present work, barium hydroxide hydrolysis for 10 hours in glass ampules always gave the highest value and was therefore used for obtaining the data on the tryptophan content quoted in the

[2] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland; L-lysine·HCl, obtained from Chas. Pfizer AB, Näsby Park, Sweden; DL-threonine, DL-methionine, DL-valine and DL-tryptophan from A.E.C., Société de Chimie Organique et Biologique, Paris, France; and L-leucine, L-isoleucine, L-phenylalanine, L-histidine from Fluka AG, Buchs SG, Switzerland.

[3] Pronase-P, California Corporation for Biochemical Research, Los Angeles.

following. The ratio between the amount of samples and the amount of barium hydroxide was found to influence the result; in our hands the optimal ratio was 3 g sample /25 ml 5 N $Ba(OH)_2$.

Pediococcus cerevisiae ATCC 8081 was used for the cystine analyses. A cup-plate procedure was applied and the medium was that developed in this laboratory by Bolinder.[4] Acid hydrolysis was used for the liberation of this amino acid. Heating 0.4 g sample in 20 ml 3 N HCl at 120° for 6 hours gave the highest yields.

RESULTS AND DISCUSSION

The amino acid compositions of the 4 rye flour mixtures, presented in table 2, are similar except for the lysine content which varies from 2.9 to 4.1 g/16 g nitrogen. Compared with wheat (3, 13, 15), rye has a higher content of both lysine and threonine, whereas the content of methionine appears to be lower. The ratio of essential to nonessential amino acids is higher in rye than in wheat flour.

A comparison of the FAO provisional pattern (16) with the amino acid analyses presented here indicates that the limiting essential amino acids for humans are, in order: methionine and cystine (53 to 60%), tryptophan (63 to 75%), lysine (67 to 95%) and isoleucine (77 to 82%). The mean protein score is 57. The amino acid composition of rye protein presented by FAO (16) gives a protein score of 80 with the sulphur amino acids as the first limiting. Vuyst and Vervack (17) also state methionine to be the first limiting amino acid and give rye protein a chemical score of 45. However, Szkilladziowa (18) and Trzebska-Jeske and Morkowska-Gluzinska (19) calculate tryptophan to be the first limiting amino acid in rye flour.

For rats, based on analytical data and the reported requirements for this species (20), the calculated order of deficiency is: lysine, methionine and cystine, isoleucine or threonine, and tryptophan (table 3). The protein level in our experiments was 8.5 to 11.5% and the fat content about 7.5%, whereas Rama Rao et al. (20), in determining rat requirements used diets containing 10% protein and 2% fat. In view of the uncertainties of both the analytical and the nutritional requirement data and the lack of information concerning the availability of the individual amino

[4] Personal communication.

TABLE 2

Amino acid composition of the 4 rye flours compared with the FAO provisional pattern of essential amino acids for humans

Amino acid	Composition in g/16 g N				Composition in g/100 g N				FAO provisional pattern
	Rye A	Rye B	Rye C	Rye D	Rye A	Rye B	Rye C	Rye D	
									g/100 g N
Alanine	4.15	4.30	4.08	3.75	25.91	26.88	25.50	23.46	—
Arginine	4.39	4.59	4.51	4.16	27.46	28.66	28.19	26.01	—
Aspartic acid	8.18	7.26	7.01	6.13	51.12	45.41	43.80	38.29	—
Cystine	1.35	1.46	1.63	1.54	8.46	9.11	10.19	9.63	
Methionine	0.94	0.99	0.95	0.98	5.89	6.22	5.95	6.13	14.4
Total sulphur amino acids	2.29	2.45	2.58	2.52	14.35	15.33	16.14	15.76	27.0
Glutamic acid	25.87	26.82	23.48	25.15	161.67	167.64	146.74	157.18	—
Glycine	4.20	4.54	4.17	4.04	26.24	28.38	26.03	25.27	—
Histidine	2.17	2.13	2.19	2.05	13.55	13.34	13.70	12.78	—
Isoleucine	3.35	3.53	3.43	3.37	20.91	22.05	21.44	21.06	27.0
Leucine	6.07	6.38	6.05	5.77	37.91	39.87	37.79	36.09	30.6
Lysine	4.13	3.41	3.16	2.88	25.80	21.31	19.76	18.01	27.0
Phenylalanine	4.30	4.56	4.39	4.26	26.85	28.49	27.44	26.65	18.0
Tyrosine	1.21	1.36	1.24	1.23	7.58	8.49	7.76	7.68	(18.0)
Proline	9.64	9.68	8.27	9.30	60.25	60.51	51.68	58.11	—
Serine	4.18	4.38	4.25	3.85	26.13	27.36	26.54	24.08	—
Threonine	3.11	3.39	3.18	3.06	19.41	21.17	19.85	19.09	18.0
Tryptophan	1.08	0.97	0.95	0.90	6.74	6.05	5.96	5.63	9.0
Valine	4.65	4.77	5.00	4.64	29.04	29.83	31.26	28.98	27.0
			Protein score		53	57	60	58	100

TABLE 3

Percentage composition of essential amino acids in diets containing 91% rye flour compared with the requirements of the growing rat

Amino acid	Basal diet containing 91% of				Required by the rat [1]	% of requirement
	Rye A	Rye B	Rye C	Rye D		
	%	%	%	%	%	%
L-Arginine	0.51	0.46	0.38	0.42	0.21	180–240
L-Cystine	0.16	0.14	0.14	0.16	0.34	41–47
L-Methionine	0.11	0.10	0.08	0.10	0.16	50–69
Total sulphur amino acids	0.27	0.24	0.22	0.26	0.50	44–54
L-Histidine	0.25	0.21	0.18	0.21	0.25	72–100
L-Isoleucine	0.39	0.35	0.29	0.34	0.55	53–71
L-Leucine	0.70	0.63	0.51	0.59	0.70	73–100
L-Lysine	0.48	0.34	0.26	0.29	0.90	29–53
L-Phenylalanine	0.50	0.45	0.37	0.43	0.42 } 0.72	67–89
L-Tyrosine	0.14	0.13	0.10	0.13	0.30 }	
L-Threonine	0.36	0.34	0.27	0.31	0.50	54–72
L-Tryptophan	0.12	0.10	0.08	0.09	0.15	53–80
L-Valine	0.54	0.47	0.42	0.47	0.55	76–98

[1] According to Rama Rao et al. (20).

acids, it would, however, not be justified to draw definite conclusions with respect to the deficiencies for either humans or rats on the basis of analytical results alone.

Table 4 shows the effect on growth, nitrogen efficiency ratio (NER) and liver fat of supplementing rye A with 7 levels of L-lysine·HCl. Maximal improvement was obtained by supplementation with about 0.25% lysine, increasing the total lysine concentration of the diet to about 0.70%. The ratio of lysine to threonine in the rye A diet is then 1.86, which is nearly the same as the ratio deduced from requirement determinations, 1.8 according to Rama Rao (20) and 2.0 according to Rose et al. (21). The addition of more lysine led to a slight impairment, but even at a level 8 times the optimal dose, growth and NER were better for the supplemented than for the nonsupplemented flour.

That lysine is the most limiting amino acid for the growing rat in all of the rye flours tested is supported by the data in tables 4, 5 and 6.

The second most limiting amino acid is threonine (table 5). Supplementation with lysine plus threonine gave an NER that was significantly higher ($P < 0.05$) than when only lysine was added. The addition of threonine alone to a rye diet did not cause a marked imbalance even at high levels (table 7), an observation which was unanticipated, especially in view of the fact that the protein value of rye flour for-

TABLE 4

Effect of lysine supplementation to rye A (nitrogen content of the nonsupplemented diet was 1.85% of the fresh weight) [1]

Group	Added L-lysine·HCl	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
	%	g/day	g/g	%
1	0	2.63 ± 0.17 [3]	12.86 ± 0.45 [3]	12.1
2	0.05	3.18 ± 0.16	13.42 ± 0.31	12.1
3	0.10	3.51 ± 0.13	14.89 ± 0.33	—
4	0.17	3.68 ± 0.16	15.56 ± 0.45	—
5	0.25	3.86 ± 0.26	16.06 ± 0.42	11.8
6	0.50	3.72 ± 0.17	15.53 ± 0.34	—
7	1.00	3.56 ± 0.18	14.93 ± 0.36	12.4
8	2.00	3.57 ± 0.25	13.80 ± 0.48	11.8

[1] Ten rats/group were fed the diets for 23 days.
[2] Liver fat was determined on a dry weight basis.
[3] SE of mean.

TABLE 5

Effect of multiple amino acid supplementation to rye C (nitrogen content of the nonsupplemented diet was 1.34% of the fresh weight) [1]

Group	Supplementation	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
	%	*g/day*	*g/g*	%
Series A				
1	—	1.52 ± 0.14 [3]	14.41 ± 0.82 [3]	9.1
2	0.3 L-Lysine·HCl	2.63 ± 0.17	19.83 ± 0.61	7.6
3	0.3 DL-Threonine	1.64 ± 0.12	14.51 ± 0.59	9.9
4	0.25 L-Isoleucine	1.80 ± 0.12	15.31 ± 0.88	10.0
5	0.5 L-Lysine·HCl + 0.3 DL-threonine	3.00 ± 0.18	21.84 ± 0.66	6.4
6	0.5 L-Lysine·HCl + 0.25 L-isoleucine	1.79 ± 0.23	15.54 ± 1.06	6.8
7	0.5 L-Lysine·HCl + 0.3 DL-methionine	2.15 ± 0.08	17.58 ± 0.27	9.4
8	0.5 L-Lysine·HCl + 0.25 L-phenylalanine	2.19 ± 0.24	17.81 ± 0.97	10.4
9	0.5 L-Lysine·HCl + 0.5 DL-valine	2.10 ± 0.12	17.78 ± 0.61	6.6
10	0.5 L-Lysine·HCl + 0.25 L-leucine	2.04 ± 0.07	16.20 ± 0.98	8.3
11	0.5 L-Lysine·HCl + 0.25 L-histidine	2.04 ± 0.20	15.72 ± 0.71	9.7
12	0.5 L-Lysine·HCl + 0.3 DL-tryptophan	1.71 ± 0.24	14.65 ± 1.50	7.4
13	Diet 5 + 0.25 L-isoleucine	2.59 ± 0.32	19.32 ± 1.64	6.2
14	Diet 5 + {0.25 L-isoleucine, 0.3 DL-methionine}	2.57 ± 0.19	20.29 ± 0.52	5.6
15	All nine amino acids above	4.05 ± 0.30	21.02 ± 0.43	8.7
Series B				
16	0.5 L-Lysine·HCl + 0.3 DL-threonine	2.87 ± 0.19 [3]	22.88 ± 0.57 [3]	8.7
17	0.5 L-Lysine·HCl + 0.5 DL-threonine	2.99 ± 0.19	23.35 ± 0.50	11.2
18	Diet 16 + 0.25 L-histidine	2.96 ± 0.12	22.65 ± 0.49	7.1
19	Diet 16 + 0.3 DL-methionine	2.89 ± 0.28	22.36 ± 0.89	8.9
20	Diet 16 + 0.25 L-phenylalanine	2.93 ± 0.24	22.13 ± 0.78	12.2
21	Diet 16 + 0.3 DL-tryptophan	2.83 ± 0.19	22.13 ± 0.73	10.9
22	Diet 16 + 0.25 L-leucine	2.98 ± 0.20	21.27 ± 0.58	11.1
23	Diet 16 + 0.5 DL-valine	2.99 ± 0.20	20.46 ± 1.02	11.4
24	All eight amino acids above	2.25 ± 0.12	18.11 ± 0.77	9.2
Series C				
25	{0.5 L-Lysine·HCl + 0.25 L-isoleucine, 0.3 DL-threonine}	2.64 ± 0.14	20.91 ± 0.43	6.9
26	Diet 25 + 0.5 DL-valine	2.42 ± 0.10	20.47 ± 0.47	9.8
27	Diet 25 + 0.5 DL-valine	2.45 ± 0.13	19.95 ± 0.45	8.4
28	Diet 25 + 0.25 L-phenylalanine	2.23 ± 0.13	19.76 ± 0.37	12.2
29	Diet 25 + 0.25 L-leucine	2.23 ± 0.17	19.41 ± 0.79	7.9
30	Diet 25 + 0.25 L-histidine	2.46 ± 0.14	19.12 ± 0.57	10.0

[1] Six rats/group in series A and B and 8 in series C: diets were fed for 21 days.
[2] On a dry weight basis.
[3] SE of mean.

tified with lysine was easily decreased by supplementation with other essential amino acids, except threonine (table 5, groups 2 and 6–12).

The third limiting amino acid was more difficult to identify. No single amino acid gave a positive effect when added to rye flour diets fortified with lysine and threonine (table 5, series A and B). The addition of a mixture of isoleucine, leucine, phenylalanine, valine, tryptophan, methionine and histidine to rye fortified with lysine and threonine improved growth. Omission of isoleucine from this mixture led to a decrease in both growth and NER (table 5, groups 15 and 24). Furthermore, the addition of even small amounts of isoleucine to a rye diet supplemented with lysine impaired growth and lowered NER (table 5, group 6 and table 8, series A). This can be taken to indicate that the deficiency in threonine, the second limiting amino acid in rye, was accentuated by increasing the content of one of the third limiting amino acids in a fashion typical of an amino acid imbalance. It was also found that this negative effect was overcome by the addition of threonine. Isoleucine, like threonine, had no deleterious effect when added to a nonsupplemented rye diet.

TABLE 6

Effect of supplementation with lysine or lysine plus threonine of rye flours, rye crisp bread and wheat flour, with isonitrogenous casein diet fed to a reference group (nitrogen content of nonsupplemented diets in series A and B was 1.34 and 1.63%)

Group	Diet	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
		g/day	*g/g*	*%*
Series A				
1	Rye flour A	1.63 ± 0.10 [3]	12.25 ± 0.29 [3]	19.8
2	Rye flour A + 0.3% L-lysine·HCl	2.28 ± 0.20	15.55 ± 0.81	13.5
3	Rye flour B	1.79 ± 0.07	13.32 ± 0.31	17.3
4	Rye flour B + 0.3% L-lysine·HCl	2.54 ± 0.15	16.70 ± 0.64	12.3
5	Rye flour C	2.09 ± 0.10	14.66 ± 0.47	13.4
6	Rye flour C + 0.3% L-lysine·HCl	2.58 ± 0.15	17.35 ± 0.67	11.9
7	Rye flour D	1.55 ± 0.08	11.51 ± 0.38	18.6
8	Rye flour D + 0.3% L-lysine·HCl	2.84 ± 0.24	17.72 ± 0.68	11.0
9	Rye crisp bread D	0.79 ± 0.04	6.82 ± 0.32	30.6
10	Rye crisp bread D + 0.3% L-lysine·HCl	2.04 ± 0.15	14.90 ± 0.67	15.8
11	Rye crisp bread D + {0.5% L-lysine·HCl, 0.3% DL-threonine}	3.31 ± 0.17	16.78 ± 0.60	8.9
12	Wheat flour	0.44 ± 0.10	4.70 ± 0.29	26.7
13	Wheat flour + 0.4% L-lysine·HCl	1.03 ± 0.08	9.28 ± 0.50	17.6
14	Casein	3.01 ± 0.07	18.98 ± 0.29	15.7
Series B				
15	Rye flour D	1.99 ± 0.11	12.68 ± 0.35	15.4
16	Rye flour D + {0.5% L-lysine·HCl, 0.3% DL-threonine}	3.64 ± 0.23	19.00 ± 0.76	13.3
17	Rye crisp bread D	1.15 ± 0.09	7.73 ± 0.47	16.1
18	Rye crisp bread D + {0.5% L-lysine·HCl, 0.3% DL-threonine}	2.88 ± 0.19	16.68 ± 0.54	13.5
19	Casein	3.67 ± 0.21	19.45 ± 0.77	16.0

[1] Eight rats/group fed diets for 28 and 21 days, respectively.
[2] On a dry weight basis.
[3] SE of mean.

TABLE 7

Effect of threonine supplementation to rye B (nitrogen content of all the diets adjusted to 1.77% of the fresh weight by the addition of glycine) [1]

Group	Added DL-threonine	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
	%	*g/day*	*g/g*	*%*
1	0	2.08 ± 0.18 [3]	11.48 ± 0.48 [3]	13.4
2	0.06	2.19 ± 0.08	11.42 ± 0.31	—
3	0.14	2.03 ± 0.13	11.66 ± 0.52	—
4	0.30	2.14 ± 0.11	10.87 ± 0.38	11.1
5	0.50	2.09 ± 0.13	10.99 ± 0.45	9.9
6	1.00	1.90 ± 0.10	10.89 ± 0.24	6.5
7	1.60	1.79 ± 0.11	10.26 ± 0.29	10.7

[1] Ten rats/group were fed diets for 22 days.
[2] On a dry weight basis.
[3] SE of mean.

No increase in the protein value was obtained by adding individual amino acids to rye diets supplemented with lysine, threonine and isoleucine (table 5, series C). It, therefore, appears that at least 3 amino acids are co-limiting after threonine. An attempt to find a group of 5 amino acids that had the same supplementary effect as the previously tested group of 9 failed (compare group 9, table 8 and group 15, table 5).

A comparison of the protein values before and after lysine supplementation of the 4 rye flour mixtures, wheat flour and a Swedish rye crisp bread is provided by the data of series A, table 6. In series B

TABLE 8

Effect of addition of isoleucine to rye C supplemented with lysine or lysine and threonine [1] *(nitrogen content of the nonsupplemented diet was 1.34% of the fresh weight)*

Group	Supplementation	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
	%	*g/day*	*g/g*	%
Series A				
1	0.5 L-Lysine·HCl	2.41 ± 0.17 [3]	17.25 ± 0.56 [3]	11.7
2	Diet 1 + 0.1 L-isoleucine	2.29 ± 0.22	16.19 ± 0.96	13.5
3	Diet 1 + 0.3 L-isoleucine	2.11 ± 0.14	15.25 ± 0.43	13.5
4	Diet 1 + 0.8 L-isoleucine	2.04 ± 0.11	14.72 ± 0.52	9.7
5	Diet 1 + 2.0 L-isoleucine	1.73 ± 0.08	13.34 ± 0.39	9.8
Series B				
6	0.6 L-Lysine·HCl + 0.6 DL-threonine	2.76 + 0.25	19.21 ± 1.02	10.9
7	{0.6 L-Lysine·HCl, 0.3 DL-Threonine} + 0.3 L-isoleucine	2.86 ± 0.23	19.71 ± 0.62	11.2
8	{0.6 L-Lysine·HCl, 0.6 DL-threonine} + 0.3 L-isoleucine	3.01 ± 0.13	19.42 ± 0.40	13.3
9	Diet 8 + {0.2 DL-methionine, 0.1 DL-tryptophan}	2.33 ± 0.22	19.02 ± 0.81	8.9

[1] Eight rats/group were fed the diets for 21 days.
[2] On a dry weight basis.
[3] SE of mean.

rye flour and the corresponding crisp bread were tested at the highest possible nitrogen concentration. The 4 rye flour mixtures did not differ markedly in the response to lysine but it is noteworthy that the largest improvement was observed with the rye having the lowest lysine content. The addition of free lysine consistently increased the growth rate and the NER and decreased liver fat, which is in agreement with the observations of Johnson and Palmer (22) and Strømnaes and Kennedy (23) who studied lysine fortification of rye flour and rye bread ingredients. Sure (24) reported only a small response to lysine supplementation. By adding valine alone he achieved an improvement that was even greater than that obtained with lysine plus threonine or with a mixture of lysine, threonine and valine. However, in the experiments described here the addition of valine to diets supplemented with lysine, lysine and threonine, or lysine, threonine and isoleucine tended to impair NER and growth.

When fed at the same nitrogen level, the rye flour diets were superior to a diet based on a 70% extraction wheat flour (table 6, series A). At the nitrogen level tested, wheat flour supplemented with lysine was still inferior to nonsupplemented rye flour. It appears likely that this is due primarily to a threonine deficiency for rats in the wheat (2, 25, 26). The superiority of rye protein over wheat protein has been reported by Mitchell and Hamilton (27) and later by Jones et al. (28), Sure (24, 29), Szkilladziowa (18, 30) and Nehring and Bock (31) using growing rats as test animals.

Although the rye crisp bread was fortified with dry milk before baking, its NER was only 60% of that of the corresponding flour (table 6, series A and B). Lysine supplementation of rye flour increased the NER 25 to 50%, whereas lysine supplementation of rye crisp bread increased this ratio about 120%. As the content of lysine in rye crisp bread D, estimated microbiologically after acid hydrolysis, was 2.9 g/16 g nitrogen or about the same as in rye flour D, it is apparent that the availability of lysine was reduced during the baking procedure. The low protein value of rye crisp bread, which also manifested itself by a tendency to cause fatty infiltration of the liver, could thus be largely compensated for by the addition of free lysine or lysine plus threonine. When supplemeted with both lysine and threonine, the NER of rye crisp bread became about 90% of that of an isonitrogenous casein diet.

Previous studies in our laboratory have indicated that the loss of added amino

TABLE 9

Comparison of the protein values of rye crisp bread, wheat crisp bread and soft white bread (nitrogen content of the 3 diets was 1.85, 1.57 and 2.03%) [1]

Group	Diet	Avg wt gain	Nitrogen efficiency ratio	Liver fat content [2]
		g/day	*g/g*	%
1	Rye crisp bread D	1.22 ± 0.11 [3]	7.24 ± 0.39 [3]	12.9
2	Wheat crisp bread	−0.02	—	26.5
3	Soft white bread	1.50 ± 0.08	7.83 ± 0.31	19.1

[1] Six rats/group fed diets for 21 days.
[2] On a dry weight basis.
[3] SE of mean.

acids during baking varies considerably from one type of bread to another and is due partly to destruction and partly to inactivation (3, 32). It would be necessary to investigate carefully the loss of added free lysine during the baking of crisp bread to evaluate the possibility of improving its nutritive value by amino acid fortification.

Table 9 shows a comparison of the protein value of rye crisp bread, wheat crisp bread and soft white bread, each fed at the highest possible nitrogen level. The wheat crisp diet did not even maintain the weight of the young rats. Likewise growth as well as NER were lower with rye crisp bread than with the soft white bread, in spite of the earlier mentioned superiority of rye over wheat flour. The baking of crisp bread evidently causes a much greater decrease in the protein value than the baking of soft bread.

Testing soft rye and wheat breads in experiments with rats, Kon and Markuze (33) obtained results indicating the superiority of rye breads and likewise Strømnaes and Kennedy (23) observed higher protein efficiency ratios for rye bread than for whole wheat bread. Kofrányi and Müller-Wecker (34) obtained similar results using the nitrogen balance technique on humans and they also noted a positive effect of lysine supplementation of rye bread. However, the approach used in their laboratory (35) makes a comparison between the biological value of the various proteins tested difficult. French and Mattill (36) noted no difference between the biological values of rye and wheat bread as determined with both human subjects and rats, and Johnson and Palmer (22) concluded that rye protein is comparable with that of wheat.

It is sometimes argued that the calculation of protein or nitrogen efficiency ratios is unnecessary (37, 38) as the correlation between these ratios and body weight gain is stated to be satisfactory. The experiments reported here do not support such a conclusion. For instance, according to the weight gain data in table 6 for groups 11 and 14, rye crisp bread supplemented with lysine and threonine would be superior to casein. The NER for the bread diet is, however, only about 90% of the ratio for casein. In a separate experiment, these 2 diets were pair-fed and in this case both weight gain and NER consistently indicated that the fortified bread diet had a protein value approximately 90% of that of the casein diet. Besides, the NER is less variable than weight gain and therefor a more useful parameter.

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Relationship between Tryptophan — Niacin Metabolism and Changes in Nitrogen Balance[1]

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ABSTRACT Two investigations of the conversion of tryptophan to niacin in adult women were made when nitrogen loss was induced by omission of lysine in one period. The normal food diet provided 10 g nitrogen, 680 mg tryptophan and 10 mg niacin. The semi-purified diet provided 10 g nitrogen, 255 mg tryptophan and 2.5 mg niacin in study 1 and 10 in study 2. A niacinamide test load indicated niacin tissue depletion in study 1 but not in study 2. A sharp increase in nitrogen retention was observed in both terminal normal food periods which followed semi-purified food periods in which there was small retention. N-Methylnicotinamide and 2-pyridone urinary excretions increased during nitrogen loss in study 2. An average of 1.7% of tryptophan was excreted as an increase in metabolites. Calculations showed that 59.2 mg tryptophan were equivalent to 1 mg niacin and efficiency of conversion was 3.0%. It is suggested that a specific percentage of tryptophan is always converted to niacin regardless of protein metabolism status.

It has been well established that tryptophan is used in supplying a portion of the niacin requirement of man and that administration of tryptophan to normal human subjects results in an increased level of urinary niacin metabolites. Vivian and co-workers (1) observed a preferential use of tryptophan in women fed diets low in niacin and tryptophan to which tryptophan was added in gradually increasing amounts. They observed that tryptophan was used first to establish and maintain nitrogen equilibrium, next for the synthesis of blood pyridine nucleotides and finally, that when the blood pyridine nucleotides had reached nearly normal levels, there was an increase in the urinary excretion of the 2 major niacin metabolites. Therefore, it was reasoned that if protein synthesis was limited by the omission of one essential amino acid, the tryptophan normally used for protein synthesis should be available from the metabolic pool for conversion to niacin and as a result the level of niacin metabolites in the urine would increase.

The present paper presents results from 2 investigations of the conversion of tryptophan to niacin in human subjects when nitrogen loss was induced by excluding lysine during one period of the semi-purified diet.

EXPERIMENTAL PROCEDURES

Subjects A total of 9 American college women participated in the investigations while engaging in their usual activities. Ages ranged from 19 to 29 years and weights were within normal range for their heights. Participants were in good health as determined by physical examinations.

Diets. The general administration of the diets and the food plans were similar to those previously described by Vivian et al. (1). In each of 2 studies (1 and 2) a control diet of normal foods[2] was given for the first 6 days. This diet provided approximately 10 g nitrogen, 680 mg tryptophan, 10 mg niacin and recommended amounts of all other known dietary essentials. In the succeeding 3 days most foods were replaced by purified components. No data were collected for this period. During the following 29 days (semi-purified regimen) a basal diet consisting of selected low protein fruits, butter

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[2] Normal foods donated by Borden's Moores and Ross, Columbus, Ohio, and The Kroger Company.

oil, cornstarch, sucrose and a vegetable oil supplied approximately 0.8 g nitrogen, 2.5 mg niacin and 25 mg tryptophan. Natural isomers of the essential amino acids (except tryptophan at 230 mg) plus arginine, histidine, cystine and tyrosine were supplied in the amounts found in 20 g of egg protein. The remainder of 10 g nitrogen was provided by glycine and diammonium citrate. Minerals and vitamins were given as supplements. A purified hemicellulose compound provided dietary bulk. Calories were adjusted to maintain a constant weight for each subject by variations in amounts of fat and carbohydrate. The semi-purified regimen was divided as follows: 9 days designated as complete; 10 days with lysine omitted from the amino acid mix; and 10 days, complete. On the last day of the semi-purified regimen a test load of 25 mg niacinamide was given orally at breakfast. In study 2 the niacin intake was 10 mg throughout the study and the study was terminated after 6 days with normal foods as compared with two days in study 1.

Methods. The 24-hour urine samples were collected under toluene, diluted to a known volume and aliquots refrigerated or frozen until analyses were completed. Daily creatinine determinations were made by means of an alkaline-picrate reagent to evaluate the completeness of collections. Feces, marked by carmine, were separated into periods and prepared for analyses. With the exception of meat, butter oil, beverages and candy, food composites equivalent to one day's diet were prepared approximately every 3 days. Meat aliquots were prepared and analyzed prior to use. The nitrogen content of aliquots of the amino acid mixes, food composites, urine and feces was determined by a macro-Kjeldhal method.

The daily urinary excretions of N-methyl-2-pyridone-5-carboxamide (pyridone) were measured by the method of Price (2) using the larger diameter columns described by Walters et al. (3). The daily urinary excretion of N-methylnicotinamide (N-Me) was measured by a modification [3] of the method of Huff and Perlzweig (4).

RESULTS

The values presented in figure 1 are averages for the last 3 days of each experimental period for all subjects in each of the 2 studies. Preliminary examination of data from these studies indicated that ranges of excretions were similar to those commonly observed in human metabolism studies and that the first few days of each period were required for adjustment to treatment.

Nitrogen balance. In study 1 the average nitrogen balance for the initial period of normal foods was 0.45 g and in the terminal 2-day period, 2.43 g (fig. 1). In study 2 nitrogen retention for the initial period was 1.48 g and for the terminal period, 1.62 g. When lysine was excluded, nitrogen loss averaged 0.76 g in study 1 and 0.50 g in study 2. In the semi-purified periods including lysine (complete) retention was observed in 3 of the 4 periods. The pattern of nitrogen storage and loss was similar in both studies except for the one period.

Pyridone excretion. The daily excretions of urinary pyridone showed a wide range among individuals during the first 3 days of both studies; thereafter the excretion levels were remarkably similar for all subjects. The individual excretion values for the 5 subjects in study 1 are presented in figure 2. The reduction in tryptophan intake from 680 mg in normal foods to 255 in the semi-purified regimen may account for the sharp decrease in excretion. The differences in levels of average excretion during the semi-purified regimen in the 2 studies are probably due to the difference in niacin intake (fig. 1). The omission of lysine resulted in an increase of excretion in both studies and when lysine was restored to the diet the excretion decreased. However, in study 2, the excretion was higher than that of the first complete semi-purified period. Following the niacinamide test load the excretion was approximately twice as great in study 2 as in study 1. During the 2-day terminal normal food period in study 1 the excretion was higher than that of the previous period, but was considerably less than that

[3] Unpublished data by the author.

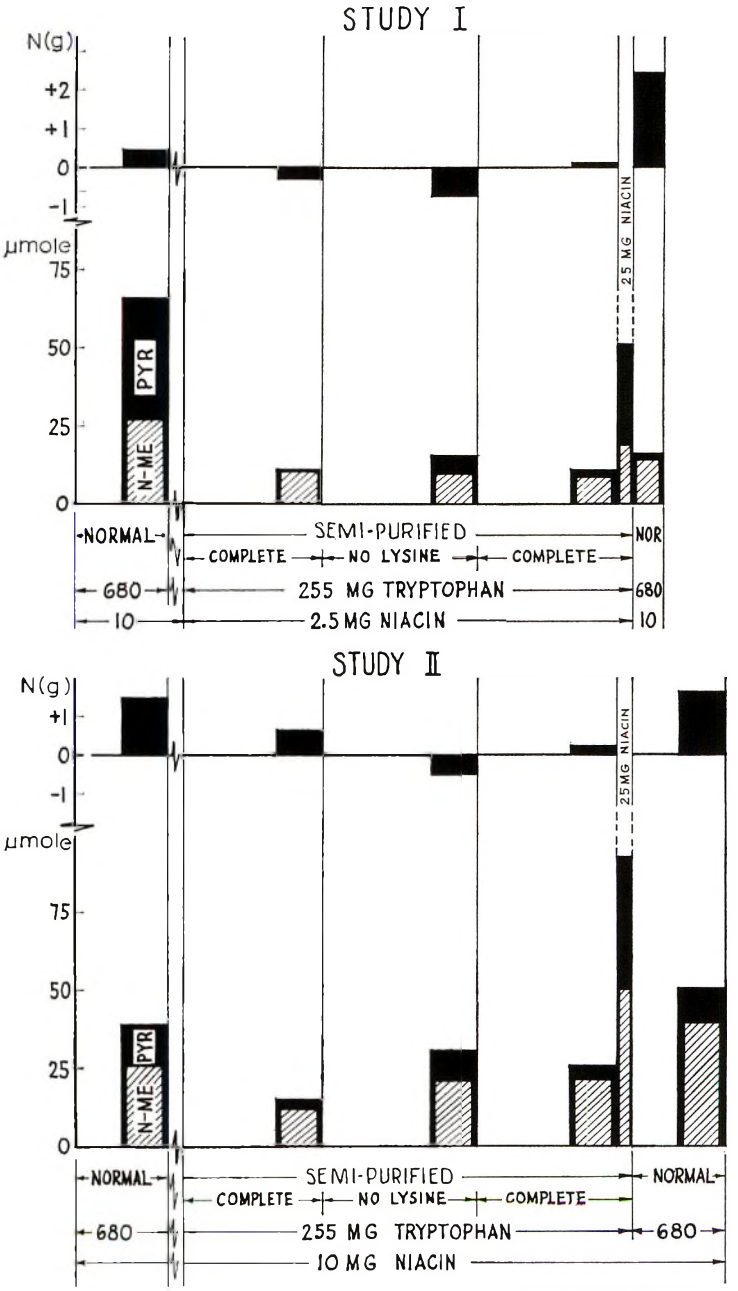

Fig. 1 Nitrogen balances and urinary excretions of N-Me and pyridone for 5 subjects in study 1, and 4 subjects in study 2. Values presented are averages for the last 3 days of each of the 5 periods except for the 1-day test load and terminal 2-day period of study 1.

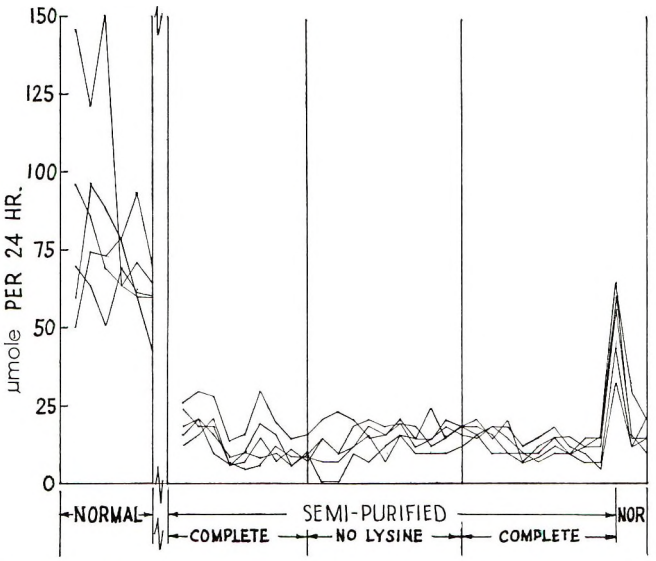

Fig. 2 Individual pyridone excretion (study 1); daily pyridone excretions of the 5 subjects.

in the initial period of normal foods. In study 2 the increase in the terminal 6-day period resulted in a level similar to that of the initial period.

N-Me excretion. Average N-Me urinary excretions in the initial normal food period and in the first complete semi-purified period were similar in both studies. In study 1 the levels of excretion were relatively constant throughout the semi-purified regimen. In study 2 the level increased in the lysine-deficient period and remained at approximately this level when lysine was restored. Following the niacinamide test load the excretion was 2.5 times greater in study 2 than in study 1. During the 2-day terminal normal food period of study 2 the excretion was higher than that of the previous period but lower than that of the initial normal food period. In study 2, the average excretion for the last 3 days of the terminal period was greater than that of the previous period as well as that of the initial period.

DISCUSSION

The nitrogen loss in the lysine-deficient period of study 1 was evidence of a limitation of normal protein tissue synthesis (fig. 1). Thus, the daily intake of 255 mg tryptophan should have been available for non-synthesis metabolic processes. However, the average pyridone excretion increased only 5 μmoles and N-Me excretion did not increase over the previous period. Furthermore, the excretion levels of both metabolites throughout study 1 were lower than those reported (5) for control subjects consuming standard diets which supplied 180 to 230 mg tryptophan and 4.3 to 6.0 mg niacin. These same workers reported that in subjects with pellagra the excretion of these metabolites was lower than the control subjects both with the standard diets and following administration of small doses of niacinamide. The low excretory levels of the subjects in study 1 and the response to the niacinamide test load were interpreted as evidence that these subjects were depleted of tissue niacin reserves. Therefore, it was assumed that any increased production of niacin from tryptophan resulted in repletion of body niacin stores rather than increased excretion of niacin metabolites.

On the basis of these results a second investigation was conducted in which the niacin intake was 10 mg for all periods. In study 2 the nitrogen loss in the lysine-deficient period was accompanied by increased excretions of pyridone and N-Me (fig. 1). This is in accord with the observations of Goldsmith et al. (6) who reported a significant increase in niacin

metabolite excretion in subjects receiving corn diets which furnished 200 mg tryptophan when the niacin supplementation level reached 8 to 10 mg. Following the test dose of niacinamide, the excretion patterns of the subjects in study 2 were similar to those of the control subjects reported by Rosenthal (5). It was concluded that there was probably no depletion-repletion of niacin reserves in study 2 and therefore the increased excretion of niacin metabolites represented the tryptophan available for conversion to niacin.

Goldsmith et al. (7) reported that an average of 3.3% of the tryptophan administered to adults receiving adequate intakes of tryptophan and niacin was converted to niacin. They also calculated that the average amount of tryptophan equivalent to 1 mg niacin was 55.8 mg (range 34 to 86). These calculations were based on excretion data of N-Me and pyridone when standard diets were supplemented independently with several levels of niacin and tryptophan. Wertz et al. (8) observed that the average amount of tryptophan equivalent to 1 mg niacin was 31 mg (range 16 to 47) in postpartum women when calculations were based on data obtained with diets supplemented with one level of tryptophan. In study 2 the calculations of the amount of tryptophan equivalent to 1 mg niacin were based on the assumption that the 255 mg tryptophan in the lysine-deficient period was completely available for conversion to niacin. Therefore, using excretion data from the first 2 periods of the semi-purified regimen, an average of 59.2 mg tryptophan was calculated to be equivalent to 1 mg niacin. Goldsmith et al. (7) also reported that an average of 1.9% of administered tryptophan and 56.9% of administered niacin were excreted as the 2 niacin metabolites. In study 2 an everage of 1.7% of 255 mg tryptophan was excreted as metabolites. Although no supplement of niacin was given in these periods, based on the assumption that these subjects would excrete at the rate of 56.9%, the percentage efficiency of conversion of tryptophan to niacin would be 3.0%. Thus it appears from the limited data in study 2 that the efficiency of conversion of tryptophan to niacin is not altered when protein synthesis is limited. This further suggests that a specified percentage of tryptophan is always converted to niacin regardless of the use of tryptophan in protein metabolism.

Vivian et al. (1) showed that even on the low levels of 25 mg tryptophan and 2.5 mg niacin there was some excretion of the 2 niacin metabolites as well as other metabolites on the pathway of tryptophan to niacin (9). Thus the perferential use of limited amounts of tryptophan reported in these studies obviously occurred after some conversion to niacin had taken place.

The fact that when nitrogen loss occurs tryptophan is released cannot be ignored. If 1 g of nitrogen was excreted this would represent about 6.25 g of protein and thus approximately 62.5 mg tryptophan. If 60 mg of this tryptophan produced 1 mg niacin, it would be difficult to measure this additional niacin as an increase in metabolites.

During all periods of the semi-purified regimens the excretion levels of N-Me were remarkably uniform for all subjects; in periods of normal foods there was a somewhat greater variation among individuals. Pyridone excretion levels followed the same pattern (fig. 2). This observation is in agreement with the suggestion of Vivian et al. (1) that when tryptophan is supplied in abundance there is variability among individuals in its metabolic utilization but a similar pattern of utilization is observed when tryptophan is limited. The greatest variation in individual nitrogen excretions (range 2.0 g) occurred in the initial normal food periods and probably reflects prestudy protein intake. Nitrogen excretions were relatively uniform in the semi-purified regimen (range in variability, 0.6 to 0.9 g).

Averages of N-Me excretions in the initial periods of both studies were approximately the same. Pyridone excretions in these periods were quite different as were the nitrogen balances. Dietary histories showed that the food intakes of the subjects prior to study 1 more nearly met the NRC recommended dietary allowances (10) than did those of the subjects in study 2. Thus, the pyridone excretion reflected the pre-study protein and niacin intakes and could be used as one criterion

of nutritional status with self-selected diets.

The sharp increase in nitrogen retention in the 2-day terminal normal food period of study 1 was not anticipated. Therefore, a 6-day terminal period was included in study 2 and the marked retention was again observed over the longer period. The nitrogen storage in each of these terminal periods followed a small storage in the preceding period. There have been indications in the literature (11, 12) that nitrogen balance is slightly better with diets of intact protein than with equivalent amounts of nitrogen from amino acid mixtures. The sharp increase in retention sustained over a 6-day span cannot be entirely explained by a difference in the source of protein. If labile protein reserves were severely exhausted in the lysine-deficient periods and only partially repleted when lysine was restored, then the additional retention of nitrogen in the terminal period might represent further repletion of labile or stable protein reserves, or both. Further investigations of this observation are underway in this laboratory.

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