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A moderate low-fat well-balanced breakfast for a man of 25 years

When a moderate reduction of dietary fat is indicated, it is worthwhile to consider a basic cereal and milk breakfast which, as shown in the table below, *contributes well-balanced nourishment. This breakfast is moderately low in fat because its fat content of 10.9 gm. provides 20 per cent of the total calories.* It provides "Men, 25 Years" with approximately one-fourth of the recommended dietary allowances[1] of protein, important B vitamins, essential minerals; and provides quick and lasting energy. The Iowa Breakfast Studies demonstrated for young men that a basic cereal and milk breakfast maintained mental and physical efficiency during the late morning hours and that it was superior in doing so when compared either to a larger or smaller morning meal.

recommended dietary allowances and the nutritional contribution of a moderate low-fat breakfast*

Menu: Orange Juice—4 oz.;
Cereal, dry weight—1 oz.;
Whole Milk—4 oz.; Sugar—1 teaspoon;
Toast (white, enriched)—2 slices;
Butter—5 gm. (about 1 teaspoon);
Nonfat Milk—8 oz.

Nutrients	Calories	Protein	Calcium	Iron	Vitamin A	Thiamine	Riboflavin	Niacin equiv.	Ascorbic Acid
Totals supplied by Basic Breakfast**	503	20.9 gm.	0.532 gm.	2.7 mg.	588 I.U.	0.46 mg.	0.80 mg.	7.36 mg.	65.5 mg.
Recommended Dietary[1] Allowances—Men, 25 Years (70 kg.—154 lb.)	3200	70 gm.	0.8 gm.	10 mg.	5000 I.U.	1.6 mg.	1.8 mg.	21 mg.	75 mg.
Percentage Contributed by Basic Breakfast	15.7%	29.8%	66.5%	27.0%	11.8%	28.7%	44.4%	35.0%	87.3%

**Revised 1958, Food and Nutrition Board, National Research Council, Washington, D.C.*

***Cereal Institute, Inc.: Breakfast Source Book. Chicago: Cereal Institute, Inc., 1959.*
Watt, B. K., and Merrill, A. L.: Composition of Foods—Raw, Processed, Prepared. U.S.D.A. Agriculture Handbook No. 8, 1950.

[1] *The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. Calorie allowances apply to individuals usually engaged in moderate physical activity. For office workers or others in sedentary occupations they are excessive. Adjustments must be made for variations in body size, age, physical activity, and environmental temperature.*

CEREAL INSTITUTE, INC.
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A research and educational endeavor devoted to the betterment of national nutrition

THE VITAMIN A REQUIREMENT OF THE YOUNG PIG [1,2]

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(Received for publication August 25, 1958)

Guilbert and Hart ('35), in appraising the vitamin A requirement of many species, concluded that it is directly proportional to body weight. Later, using growth, the degree of nyctalopia in farm animals, and cornification of vaginal smears in rats as criteria for adequacy, these workers (Guilbert et al., '40) arrived at the conclusion that for rats, swine, sheep, cattle and horses, the requirement ranged between 3.8 and 6.4 μg of vitamin A/kg of body wt./day. About three times the minimum was required for significant liver storage and optima of reproduction and dark adaption.

Braude and associates ('41) considered the minimal requirement of the pig from 8 weeks of age and older to be 100 I.U. of vitamin A/10 lb. live wt./day. Later, the need of the pig of about the same age for a purified source of carotene (Hentges et al., '52) was determined to be 25 μg/kg of body wt./day. This resulted in some liver storage. Forty micrograms were necessary for a normal blood content, although 10 μg produced satisfactory growth. The measurement of the spinal fluid pressure, as a criterion for adequacy, was developed by Moore and Sykes ('40). It was observed by them that the pressure rose at about the same stage of insufficiency as that

[1] Journal Paper no. J-3377 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 959.

[2] Grateful acknowledgment is extended to Hoffmann-La Roche, Inc., Nutley, New Jersey and Western Condensing Company, Appleton, Wisconsin for grants-in-aid and materials which partially supported this research.

at which nyctalopia became apparent. In a technique of liver biopsy suitable for mature gilts (Jones et al., '56), samples from the liver margin were shown to give a satisfactory picture of the total content.

It was pointed out by Guilbert and Hart ('35) that if the requirement is proportional to body weight, the quantity needed per pound of feed should increase with size as energy requirement and feed consumption increase approximately as the 0.74 power of body weight. However, as younger animals have a faster growth rate, compensation for this effect should mitigate any differential in the requirement. Arnrich and Morgan ('54) concluded that both body weight and growth are the important factors in determining the requirement, and that metabolic rate is of little consequence.

The pig is born relatively deficient in vitamin A so that there is an initial need for higher quantities of this vitamin to alleviate this deficit. Normally the pig accumulates considerable quantities during the first week of life from the colostrum (Liu et al., '56).

This present paper describes 4 experiments, involving 224 pigs, designed to study the vitamin A requirement of the pig up to 8 weeks of age, since early weaning partially excludes the sow as a source of the vitamin to the young pig. The response to the diet was measured by blood plasma and liver vitamin A levels, weight gains, feed efficiency, cerebrospinal fluid pressure and gross clinical and histological symptoms of deficiency.

EXPERIMENTAL

For each of the 4 experiments mature crossbred sows were fed a vitamin A-low ration from shortly after breeding until the weaning of their pigs at one week of age.

Experiment 712. Milk samples were collected from three of the sows in experiment 712 and two comparable sows receiving the normal herd ration on the first, 4th and 7th days of lactation and the vitamin A and carotene contents were determined by the antimony trichloride reaction using a Bausch and Lomb colorimeter.

Three pigs were placed in each of 8 pens, giving a total of 24 pigs. They were fed a vitamin A-deficient prestarter from one until 4 weeks of age at which time they were repleted on a starter ration with vitamin A[3] being administered by stomach tube twice weekly according to body weight at the time of dosage.

Blood plasma vitamin A was determined at the start, middle and close of the repletion period by the method of Kimble ('39). Liver vitamin A analysis by the method of Gallup and Hoefer ('46) was carried out at the close, and cerebrospinal fluid pressures were determined at the start and close of the repletion period.

To obtain liver samples in these experiments, a biopsy by laparotomy was developed in which a 1.5- to 2.5-gm sample was taken from the tip of the right central lobe. The left central lobe was sampled in older pigs. This method was found to be successful in all animals between the ages of 6 days and 9 weeks. In some cases samples were removed at one week of age and again at three weeks. It was shown in an earlier experiment that the method had very little influence upon growth rate.

Cerebrospinal fluid pressures were measured by a puncture of the subarachnoid space through the dorsal opening of the atlanto-occipital articulation. The pig was laid horizontally on its side while the reading was taken as the average over a period of several minutes. For both this measurement and for the biopsy, sodium pentobarbital was used as an anaesthetic.

Experiment 725. Eighty pigs (20 pens of 4 pigs/pen) were fed a diet containing various levels of vitamin A[4] in the diet from one until 8 weeks of age. The various physiological

[3] This vitamin A preparation was a specially prepared water dispersion by Hoffmann-La Roche, Inc., Nutley, New Jersey. It contained 200,000 U.S.P. units of vitamin A/gm, as vitamin A palmitate. Also included were 2 mg of DPPD/gm of dispersion. The solution was diluted to the required levels with distilled water and stored in brown glass bottles at 0°C during the course of the experiment.

[4] Rovimix, prepared by Hoffmann-La Roche, Inc., Nutley, New Jersey.

measurements were made at 8 weeks of age in experiments 725, 743 and 779.

Heat lamps were used in this and the previous experiment to provide restricted space heating.

Experiment 743. Thirty-six pigs were individually fed in pens supplied with radiant floor heating. Again the vitamin A was offered at various levels in the diet from one until 8 weeks of age.

Experiment 779. Eighty pigs were housed (4 pigs/pen) in a warm environment until three weeks of age when the heat lamps were removed. Up until this age the pigs received a vitamin A-deplete diet but were then transferred to a diet containing different levels of dry vitamin A for the remainder of the experiment. At 4 weeks of age half the pigs were subjected to a temperature which was rapidly stabilized at 7°C with a relative humidity which varied between 70 and 100%. The other half of the animals were reared in a temperature initially at 21°C and this was gradually lowered to 17°, (relative humidity 25 to 70%).

Experiments 712, 725 and 743 were randomized block designs and 779 was a split plot design. The animals were allotted to pens by outcome groups of initial weight within litter, with control on sex being obtained in experiment 779. All male pigs were castrated at about 4 days of age. Ration formulas are presented in table 1.

RESULTS

The gestation depletion ration produced a marked effect both upon the vitamin A content of the colostrum and upon the liver storage in the pigs suckled by these sows. The influence upon the carotene content of the colostrum was much less obvious. These trends are illustrated in figure 1.

In the earlier experiments, the obvious vitamin A deficiency symptoms did not appear in the pigs fed the basal ration until about 4 weeks following weaning. These included a ruffling of the hair coat on the forehead, change in guttural tone and xerophthalmia. However, obvious keratinization of the cornea

TABLE 1

Composition of basal rations

INGREDIENT	EXP. 712	EXP. 725 [1]	EXP. 743, 779 [2]	
	4 to 9 wk.	1 to 5 wk.	1 to 5 wk.	5 to 8 wk.
Dried skim milk (low heat, spray-dried)	35.0	40.0	40.0	20.0
Sucrose	10.0	15.0	16.0	15.55
Lactose	—	5.0	4.89	—
Dextrose	22.24	7.07	—	—
Ground white corn	—	—	10.0	42.0
Solvent soybean oil meal (50% protein)	12.0	—	—	14.0
Soy protein [3]	—	9.3	7.37	—
Dried brewers' yeast	2.0	2.0	2.0	2.0
Stabilized lard	3.0	4.0	4.0	—
Dried beet pulp	2.0	2.0	2.0	2.0
Dried whey (70% lactose)	10.0	10.31	10.31	—
Corn steep water	—	2.0	—	—
Iodized salt	0.5	0.5	0.5	0.5
Dicalcium phosphate	1.11	0.67	0.78	1.80
Trace mineral mixture [4]	0.15	0.15	0.15	0.15
Vitamin-antibiotic premix [5]	2.00	2.00	2.00	2.00
Totals	100.00	100.00	100.00	100.00

[1] The ration fed from 5 to 8 weeks of age was the same as in Experiment 743 at that age.

[2] For Experiment 779 the ration was changed at three weeks of age instead of 5 weeks.

[3] Drackett Assay Protein C-1.

[4] Contributed the following minerals as percent element in mixture: Fe, 7.00; Cu, 0.47; Co, 0.17; Zn, 8.10; Mn, 5.68; Ca, 5.28; K, 0.75.

[5] Each 2 lb. of premix contained the following amounts of vitamins and antibiotics for pigs from 1 to 5 weeks of age or from 4 until 9 weeks: vitamin D_2, 100,000 I. U.; α-tocopheryl acetate, 0.3 gm; menadione, 0.05 gm; riboflavin, 0.02 gm; Ca pantothenate, 0.08 gm; niacin, 2.25 gm; vitamin B_{12}, 2 mg; folic acid, 0.05 gm; ascorbic acid, 10.0 gm; thiamine HCl, 0.3 gm; pyridoxine, 0.15 gm; *p*-aminobenzoic acid, 0.20 gm; inositol, 25.0 gm; choline chloride, 10.5 gm; chlortetracycline, 4.0 gm; penicillin, 4.0 gm; streptomycin, 2.0 gm.

Each 2 lb. of premix contained the following amounts of vitamins and antibiotics for pigs from 5 to 8 weeks of age: vitamin D_2, 50,000 I.U., riboflavin, 0.2 gm; Ca pantothenate, 0.08 gm; niacin, 2.25 gm; vitamin B_{12}, 1.5 mg; choline chloride, 8.5 gm; chlortetracycline, 2.0 gm; penicillin, 2.0 gm; streptomycin, 1.0 gm.

only appeared in one pig, which was in an earlier experiment not reported here. Differential growth rate was detectable in the most recent experiment (779) before 4 weeks of age. The most striking deficiency symptom, which developed between 7 and 8 weeks of age, was acute paralysis of the hind quarters, particularly noticeable in those pigs which maintained a fairly

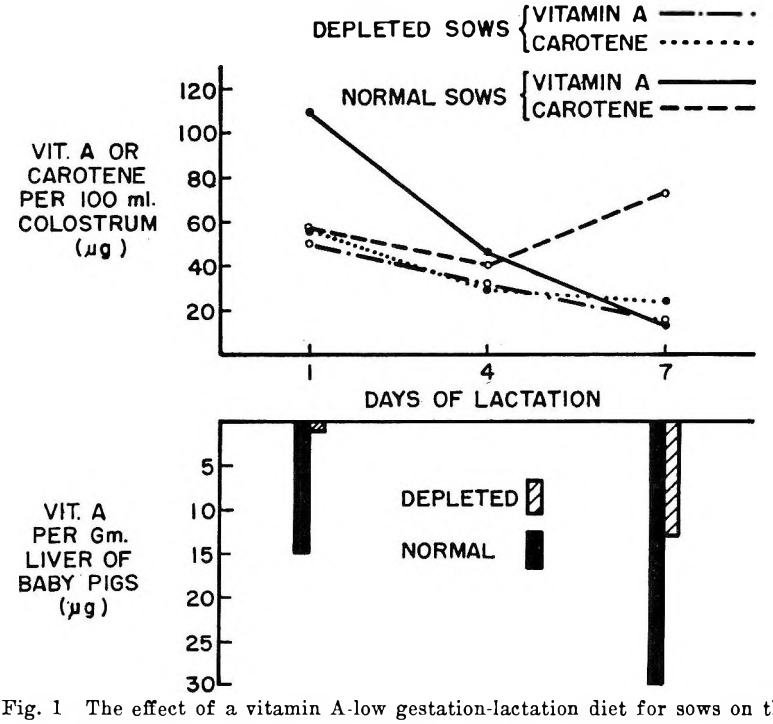

Fig. 1 The effect of a vitamin A-low gestation-lactation diet for sows on their colostrum vitamin A and carotene content and the liver vitamin A content of their young.

rapid rate of gain. Food consumption continued in this condition and, initially, periods of apparent recovery from the paralytic condition occurred. No differential effect of vitamin A on sex was noticed in any measurement. Massive oral doses of vitamin A were found in many cases to be effective in bringing about recovery in these pigs after 8 weeks of age. Death resulting from this extreme deficiency was rare even when pigs were left in such a state until 15 weeks of age.

TABLE 2

Summary of average gains and feed efficiency as influenced by dietary vitamin A in all experiments

VIT. A ADDED	EXP. 712	EXP. 725		EXP. 743		EXP. 779			
						Av. daily gain [3]		Lb. feed per lb. gain	
	Av. daily gain [1]	Av. daily gain [2]	Lb. feed per lb. gain	Av. daily gain [2]	Lb. feed per lb. gain	High temp.	Low temp.	High temp.	Low temp.
I.U.	*lb.*	*lb.*		*lb.*		*lb.*	*lb.*		
0	0.67	0.72	2.02	0.77	1.85	0.50	0.48	1.95	2.07
7 [4]	0.88								
14.5		0.69	1.87						
49 [4]	0.94								
55		0.86	1.85	0.80	1.79				
100						0.75	0.86	1.89	1.88
107		0.91	1.89						
208		0.87	1.86	0.93	1.82				
343 [4]	0.93								
400						0.83	0.92	1.93	1.91
790		0.90	1.95	0.97	1.83				
1540				0.96	1.93				
1600						0.96	1.03	1.95	1.97
3000		0.86	1.96	0.93	1.92				
6400						0.97	0.96	1.85	1.97
11393				0.94	1.88				

[1] Av. daily gain between 4 and 8.5 weeks of age.
[2] Av. daily gain between 1 and 8 weeks of age.
[3] Av. daily gain between 3 and 8 weeks of age.
[4] I.U. per lb. body wt. per day. Remainder are I.U. per lb. of feed per day.

Weight gain and feed efficiency data at 8 weeks of age are shown in table 2. Differences referred to as significant pertain to a $P < 0.05$ whereas those referred to as highly significant pertain to a $P < 0.01$. The regressions of gain on log dose were statistically significant in experiments 725, 743 and 779 (linear in 725 and quadratic in 743 and 779). Maximum gain occurred between the limits of 100 and 1600 I.U. of vitamin A/lb. of feed. The slight depressions in gain at the two highest levels compared to the next two levels in experiment 743 were greater than the least significant difference. The slightly greater observed rate of gain at the lower temperature in experiment 779 was not significant, but the design of the experiment was such that it gave only a poor estimate of error for measuring the main effect of temperature. The interaction of temperature and vitamin A on growth approached statistical significance, suggesting an increased requirement at the higher temperature. In all experiments very little vitamin A was necessary to elicit a near normal rate of gain.

The plot of feed efficiency observed in these three experiments was found to be curvilinear, being a highly significant quadratic regression in experiment 725. Some light will be thrown upon this effect in a later paper. Carcass analyses of 14 of the pigs from experiment 725 gave no hint of a trend with respect to specific gravity. Thyroid gland weights per pound of body weight rose to a maximum at 790 I.U. of vitamin A and declined in pigs receiving higher levels of the vitamin.

DISCUSSION

The vitamin A requirement for weight gain appeared to be slightly less and more inconsistent than the requirement for normality in the other three main physiological criteria (spinal fluid pressure and liver and plasma vitamin A).

It was only the highest losage level (343 I.U. vitamin A/lb. of body wt./day) in experiment 712 which was sufficient to return previously pathological pressures to normal. This may have been a consequence of too short a repletion period (4

weeks) to allow the previous condition to be alleviated at marginal vitmain A levels. Further, an overly long initial depletion not only allowed the animals to become depleted of their reserves, but also permitted the pathological conditions, as indicated by abnormal pressures in all groups at 4 weeks of age, to proceed so far that the subsequent dietary vitamin A was acting as a therapeutic agent.

Before any final conclusion may be drawn as to the requirement under the conditions tested, it is important to consider the legitimacy and precision of the various criteria used to estimate the dietary adequacy. Furthermore, the trends suggested by each criterion must be capable of interpretation.

For meat production weight gain is the most valid criterion of adequacy if the level of vitamin A leading to maximum gain would result in highest gains under any environmental condition that the pig is likely to encounter in practice. However, maximum gain was not always accompanied by normality in other bodily functions casting doubt on the universal applicability of gain as a good criterion.

The precison of a criterion within one experiment may be estimated by taking into account the steepness of the slope of the response curve and the deviations of the observations about the mean response line. With this end in view, use was made of the lambda value suggested by Bliss (in György, '51) for biological assay. Responses only up to and including 790 I.U. of vitamin A/lb. of feed were utilized in experiment 725. Similarly for experiments 743 and 779, data only up to and including 1560 I.U. and 1600 I.U., respectively, were used. Growth, plasma vitamin A and spinal pressure were considered. Plasma vitamin A was shown to be the most sensitive criterion in each of the three experiments. Spinal pressure was seen to be the least sensitive in experiments 725 and 779, but gain the least in experiment 743. The lambda values, as averages of all three experiments, indicated plasma to be the most sensitive and gain in body weight the least.

The level of response desired must be readily definable. An inflection point of the plasma response curve on log dose can

be detected in all experiments, with an average value at 17 to 18 μg of vitamin A/100 ml of plasma, according to the analytical method used. Since this is the point at which the spinal fluid pressure reached generally accepted normal values, the inflection point can be considered a valid one representing the attainment of normality. However, inflection points are difficult to define closely from most biological data even after transformations. In comparison, at this same dietary level the spinal fluid pressure response shows a sharp break in the

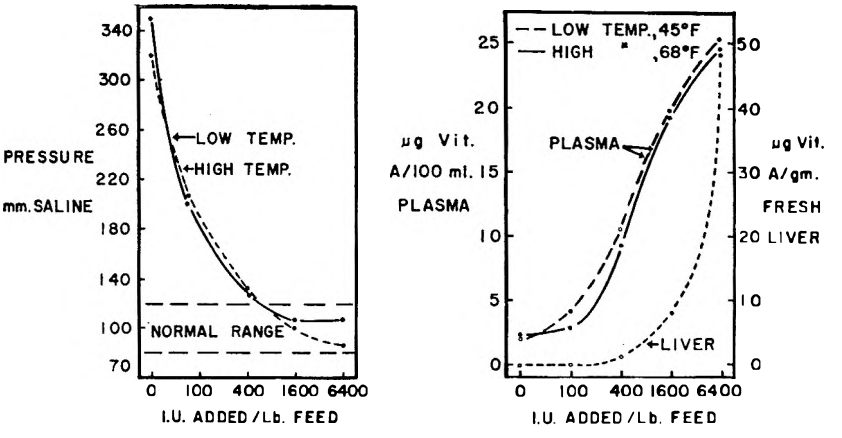

Fig. 2 Effect of dietary vitamin A and ambient temperature on (A) the cerebrospinal fluid pressure and (B) plasma and liver vitamin A at 8 weeks of age.

curve of response on log dose. In fact, at higher dietary levels, the response remains relatively constant (fig. 2 and table 3).

Although the consistency between experiments was fairly high with plasma values, the weight gain response and its maximum point varied considerably between experiments.

It was noticed that a detectable liver storage of vitamin A corresponded almost exactly to the same dietary vitamin A level (800 I.U./lb. of feed) in experiments 712, 725, 743 and 779. This was the level at which the other criteria investigated attained normality. Thus when sufficient of the vitamin is

TABLE 3

Summary of cerebrospinal fluid pressure, plasma and liver vitamin A at 8 weeks of age as influenced by dietary vitamin A

VIT. A ADDED	EXP. 712			EXP. 725			EXP. 743		
	Pressure [1]	Plasma [2]	Liver [3]	Pressure [1]	Plasma [2]	Liver [3]	Pressure [1]	Plasma [2]	Liver [3]
I.U.									
0	275	5.2	0.0	399	0.9	0.1	258	1.5	0.0
7 [4]	219	5.5	0.0						
14.5				335	1.4	0.1			
49 [4]	166	16.4	4.0						
55				338	2.5	0.2	203	4.0	0.3
100									
107				251	4.5	0.1			
208				165	5.6	0.2	140	7.7	0.1
343 [4]	114	20.5	51.5						
400									
790				121	15.8	3.1	83	21.3	3.0
1540							100	17.8	7.7
1600									
3000				122	20.5	24.4	99	24.3	24.4
6400									
11393							105	29.6	123.0

[1] Cerebrospinal fluid pressures in millimeters of saline.

[2] Blood plasma vitamin A in μg per 100 ml plasma.

[3] Liver vitamin A in μg per gm fresh tissue.

[4] I.U. per lb. body wt. per day. Remainder are I.U. per lb. of feed per day.

provided in the diet to supply adequately all bodily functions, any surplus is stored in the liver. Since all these animals initially had a negligible reserve in the liver, the occurrence of liver storage indicated that the diet was at least on the borderline of adequacy. This can then be considered a very precise criterion in the pig for vitamin A adequacy.

If the liver response is consistent between experiments, it can be logically argued that the requirement for vitamin A is

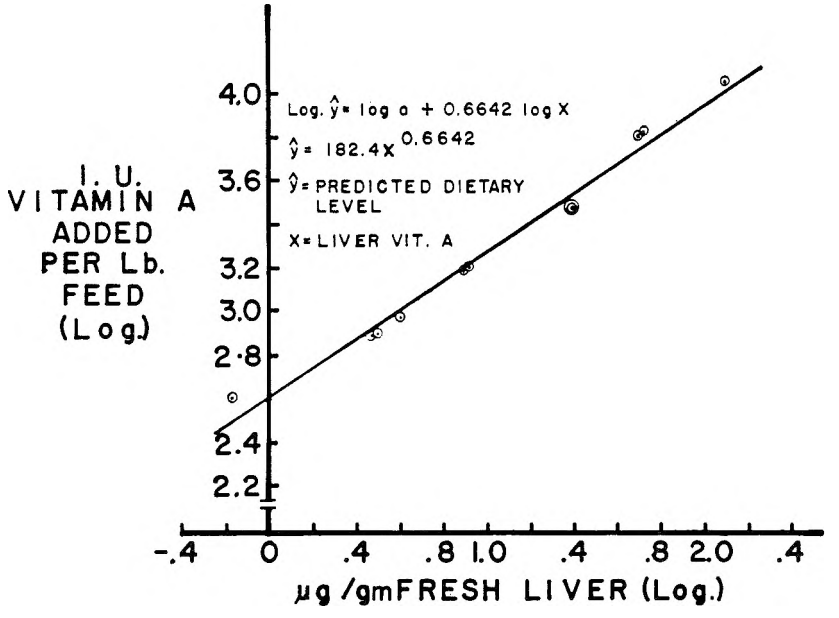

Fig. 3 Relationship between liver storage of vitamin A and dietary level. A summary of 4 experiments over two years, involving 226 pigs.

at least not influenced to any great extent by uncontrolled variations in management, season and diet experienced in these experiments. This evidence is supported by the slight effect of the temperature difference imposed in experiment 779 (see A in fig. 2). This point is brought out in a more lucid fashion in figure 3 where liver response is plotted for the complete dietary range tested in all 4 experiments, from the point of minimal detectable storage. Each point represents

the mean response to one dosage level in one experiment. Liver storage is shown to vary as a power function of the dietary content. Other workers with cattle (Frey et al., '47) observed a linear response when the values along both axes were in arithmetic intervals.

In order that the liver response curve might be used in the determination of biological values for any vitamin A or carotene source for the young pig, the appropriate prediction equation was derived. This is recorded in figure 3.

An earlier investigation not reported here, where pigs were fed a vitamin A-deplete diet from 6 days of age, and in which these pigs were biopsied at 6 days and again at 20 days, suggested that the rate of utilization of stored liver vitamin A was equivalent to 800 I.U./lb. of feed (assuming 100% utilization of the dietary vitamin A). This would suggest that the true dietary need is somewhat higher at this early age. The situation in 14-week-old pigs, to be discussed in a later paper, suggested that the requirement has dropped below that apparent at 8 weeks of age. The requirement as estimated by the authors can be compared approximately with that obtained by other workers converting their values to the same denomination. The conclusions of Guilbert et al. ('40) approximate 200 I.U. of vitamin A/lb. of feed as the average minimum for all species with three times this being required for significant liver storage and optima of reproduction and dark adaption. For pigs over 8 weeks of age the work of Braude et al. ('41) suggests 250 I.U. of vitamin A/lb. of feed and that of Hentges et al. ('52) suggests 475 I.U. of purified carotene/lb. of feed to be the minimum required. These values are somewhat less than those obtained by the authors for the pig between one and 8 weeks of age.

SUMMARY

Four experiments involving 226 pigs carried out over a period of two years have been described. These pigs were from sows reduced considerably in their vitamin A reserves by previously feeding a ration low in vitamin A. They were

weaned at 7 days of age and the requirement for vitamin A was estimated in the first 8 weeks of life. Several criteria of adequacy were investigated and their sensitivity, precision and validity discussed. These criteria were weight gain, feed efficiency, bloood plasma and liver vitamin A, and cerebrospinal fluid pressure. Other functions also studied will be discussed in later papers.

Of the three criteria (weight gain, plasma vitamin A and spinal fluid pressure), the plasma response was the most precise and the weight gain the least as measured by the lambda value of Bliss (in György, '51). However, the minimum point of normality was most readily detectable in the spinal fluid pressure response and this point occurred at a similar dietary intake of vitamin A in all experiments (600 to 800 I.U.). The liver vitamin A accumulation, estimated on fewer animals, was found to be extremely sensitive and very consistent between experiments.

The minimum requirement of the young pig for a stabilized source of vitamin A palmitate on a dry carrier was judged to be 800 I.U./lb. of feed under the conditions which existed. Normality in weight gain occurred at as low as 100 I.U./lb. of feed. Acute paralysis of the hindquarters was the most striking deficiency symptom.

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RELATIONSHIP OF VITAMIN A TO S^{35} METABOLISM IN THE BABY PIG [1]

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(Received for publication August 25, 1958)

Mellanby ('44) found that in a deficiency of vitamin A, the overall sizes of the vertebrae are the same as in normal animals, but the spinal canal is less in diameter, thus making the bone thicker. This could induce a hydrostatic pressure upon the cerebrospinal fluid. However, other workers (Woollam and Millen, '56) suggest that a vitamin A-deficient regime is causal in the increased production of cerebrospinal fluid in the chick. General appraisal of the work of Wolbach and Bessey ('41) and of Mellanby indicates that in all ages of rats and dogs periosteal overgrowth is a feature of avitaminosis A. However, the earlier in life that deficiency occurs, the more probable is the supervention of inanition and cessation of epiphyseal growth.

Dziewiatkowski ('54) demonstrated an increase in the uptake of injected inorganic radioactive sulfate by epiphyseal cartilage when vitamin A-depleted rats received the vitamin. He proved that this accumulation occurred as chondroitin sulfate. In this circumstance the chondroitin sulfate may act as a cation binder, retaining calcium in preparation for its sub-

[1] Journal Paper no. J–3398 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 959.

[2] Acknowledgment is made to Hoffmann-La Roche, Inc., Nutley, New Jersey for grants-in-aid and materials which partially supported this research, and to Mr. Lou Facto of the Iowa Agricultural and Home Economics Experiment Station for assistance in producing the radioautographs.

sequent release and more stable precipitation in the presence of an abundance of phosphate ions. Alternately it may act as a template or mould in the deposition of apatite crystals (Kent et al., '56).

Fell et al. ('56), working with chick bone rudiments *in vitro*, found that the intracellular material of cartilage decreased in size and then tended to disintegrate in the presence of excess vitamin A. These workers found that radioactive sulfate was bound, in normal cartilage, to an organic substance which was synthesized in the flat proliferating and young hypertrophic cartilage cells, and which then diffused into the matrix. Chondroitin sulfate was continuously catabolized in the matrix and replenished from the cells.

The present study was undertaken (1) to determine the rate at which an intraperitoneal injection of $Na_2S^{35}O_4$ was absorbed and utilized by the various tissues of the pig; (2) to study the effect of different levels of dietary vitamin A, within a range which would be experienced in practice, upon the S^{35} metabolism in the tissues of the pig by slaughtering the animals at the point of maximum tissue accumulation of S^{35}; (3) to determine the relationships between cerebrospinal fluid pressure and sulfur metabolism of the costochondral junction in vitamin A deficiency; and (4) to investigate the histology of the parotid salivary and osteoid tissues of the pig.

EXPERIMENTAL

Experiment 688. To study the rate of absorption and distribution of intraperitoneally injected $Na_2S^{35}O_4$, 5 normal pigs of similar age, condition, breeding and weight (average initial weight, 4.5 kg) were selected and fed individually the diet in table 1 in metal-walled pens with wire-screen bottoms. They were allotted at random to the 5 treatments, which were 5 sequential times of injection (0.2 microcuries/gm of body wt.) of carrier-free $Na_2S^{35}O_4$ prior to slaughter. Tissue samples were analyzed for S^{35} by a modification of the method of Cember et al. ('54). The barium sulfate precipitates were washed on sintered glass filters covered with Whatman No. 50 filter papers.

Radioautographs were prepared of 270 longitudinal sections (7 microns thick) of the costochondral junctions of the 4th and 5th ribs. The bones were fixed in a 3.7% formalin for 24 hours and then decalcified in either a 10% sodium versenate solution (pH 7.5) or 3% HCl in 70% ethanol (pH 1.0). Five sections from each decalcifying treatment within each time

TABLE 1

Composition of ration

INGREDIENT	AMOUNT
	lb.
Dried skim milk (low heat, spray-dried)	40.0
Cane sugar	10.0
Dextrose	5.0
Ground yellow corn	9.6
Solvent soybean oil meal (50% protein)	17.5
Dried brewers' yeast	1.0
Stabilized lard	5.0
Dried beet pulp	2.0
Dried whey (sweet)	2.5
Condensed fish solubles	2.5
Corn steep water	1.0
Iodized salt	0.5
Dicalcium phosphate	0.6
Calcium carbonate	0.15
Trace mineral mix [1]	0.15
Vitamin-antibiotic premix [2]	2.50
Total	100.00

[1] Contributed the following minerals as percent element in mixture: Fe, 7.0; Cu, 0.47; Co, 0.17; Zn, 8.10; Mn, 5.68; Ca, 5.28; K, 0.75.

[2] Each 2.5 lb. of premix contained the following amounts of vitamins and antibiotics: vitamin D_2, 60,000 I.U.; vitamin A, 980,000 I.U.; riboflavin, 0.3 gm; Ca pantothenate, 0.35 gm; niacin, 1.8 gm; vitamin B_{12}, 2 mg; folic acid, 0.9 gm; choline chloride, 25.0 gm; chlortetracycline, 10.0 gm.

treatment were placed on each of 5 or 6 microscope slides. The slides were grouped together with lead backing and were covered with a sheet of thick black paper. Above the paper was placed an 8 in. × 10 in. sheet of Contrast Process Orthofilm.[3] This was exposed at room temperature to the emanations for a period of 14 days. In order to standardize densi-

[3] Eastman Kodak, Rochester, N. Y.

ties, the films were developed simultaneously for two minutes in Dektol,[3] diluted 2 to 1 with water. Optical densities of the line of maximum activity on the autographs were measured with a Weston Photo Analyzer, Model 877.[4]

Experiment 755. A study was made of the influence of various dietary vitamin A levels (ranging from 0 to 11,400 I.U. per lb. of diet) on the distribution of intraperitoneally injected S^{35} in various pig tissues. Seven pigs, 8.5 weeks of age, were fed in the pens described above (Frape et al., '59, exp. 743 — ration 5 to 8 weeks of age). They were sacrificed 17 hours post-injection. The thyroid and parotid glands were removed for histological study with longitudinal sections of rib costochondral junctions.

Six microscope slides of 5 sections per slide were prepared for each pH of decalcification in each ration treatment. Instead of being covered with black paper, the sections were sprayed with acrylic plastic[5] to prevent pseudoradioautographic effects.

Tissues were analyzed for S^{35} using another modification of the method of Cember et al. ('54). The barium sulfate precipitate from each tissue sample was centrifuged in tubes cut from plexiglass tubing coated internally with "Desicote"[6] and fitted with removable bottoms, which were stainless steel planchets 2.441 cm average internal diameter. Blood plasma, instead of whole blood as in experiment 688, was analyzed for S^{35}.

The procedures used for measuring cerebrospinal fluid pressure and plasma and liver vitamin A concentrations are described by Frape et al. ('59).

RESULTS AND DISCUSSION

Experiment 688. The maximum S^{35} activity in soft tissues occurred at approximately 9 hours or less after intraperi-

[3] Same as footnote 3, see page 191.

[4] Western Electrical Instrument Corporation, Newark, New Jersey.

[5] Krylon, (Crystal Clear, no. 1301), plastic aerosol spray, Krylon, Inc., Philadelphia 46, Pennsylvania.

[6] Beckman Instrument, Inc., South Pasadena, California.

toneal injection, whereas maxima were at 16 to 18 hours for cartilage and rib junctions (fig. 1). Further, the S^{35} accumulation in the latter tissues was of the order of 10 times that in the soft tissues. The S^{35} activity in the costochondral junction also was indicated by the optical densities of the radioauto-

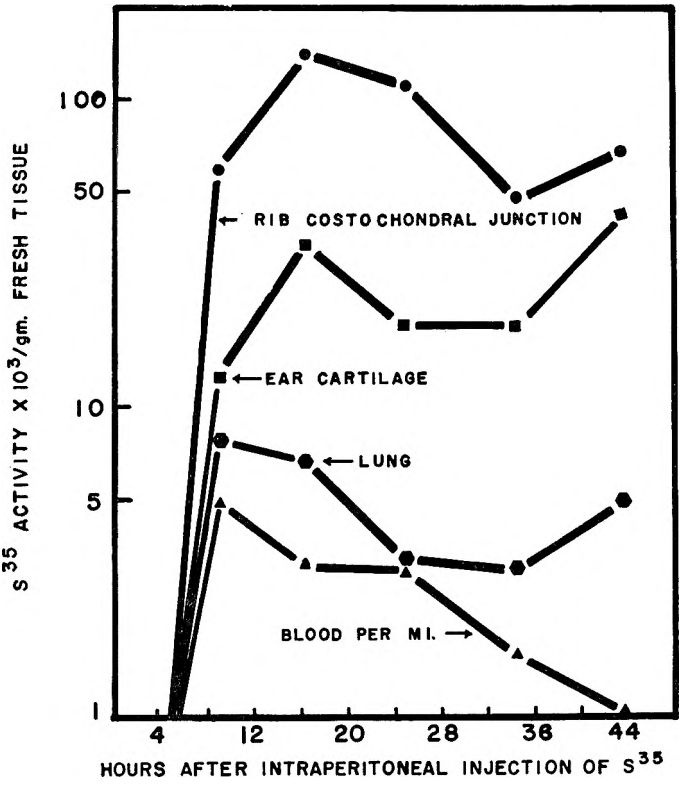


Fig. 1 Experiment 688. S^{35} activity of various tissues of 5 pigs one slaughtered at each time interval following intraperitoneal injection of a carrier-free solution of $Na_2S^{35}O_4$. The diet contained 9800 I.U. of vitamin A/lb.

graphs at the various times (fig. 2). The organic sulfate appeared to be more soluble in sodium versenate at pH 7.5 than in HCl at pH 1.0, since the activity was less in sections decalcified by the former. The region of the S^{35} accumulation was seen to be principally that of the hypertrophic cartilage cells and also that of the adjacent zone of proliferation.

For the statistical study of the radioautographs, 5 slides with 5 sections per slide were used for each of the subtreatments. The optical densities of the line of maximum activity in the costochondral junction were investigated by an analysis of variance. This indicated that if the number of sections per slide were increased to 6, the precision would be increased by 9.8%. However, if instead, the number of slides per treatment were increased to 6, the precision would have been increased

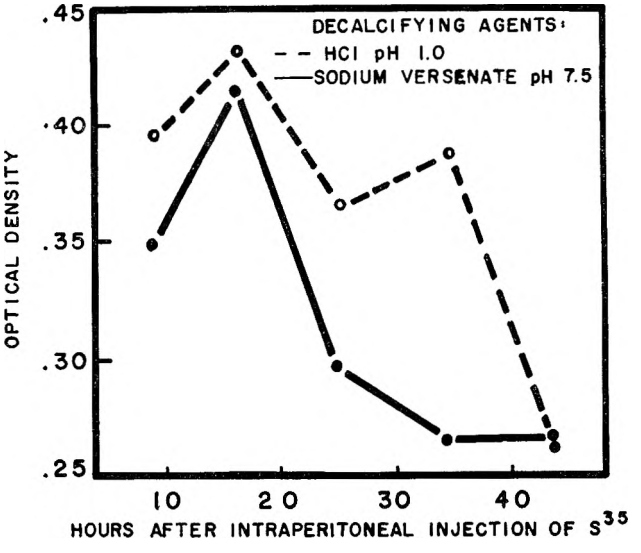


Fig. 2 Experiment 688. S^{35} activity of the 4th rib costochondral junctions as measured by the optical density of radioautographs of 7 micron longitudinal sections decalcified by two reagents.

by 20%. Advantage was taken of this information in the design of the radioautographic methods employed in experiment 755.

Experiment 755. The maximum accumulation of S^{35} in the rib junction occurred at 17 hours in experiment 688, at which time the activity in the lung and blood had not dropped appreciably. This maximum point was shown by Dziewiatkowski ('54) to be the region in which the greatest difference in sulfur activity might be expected between groups receiving and

those not receiving vitamin A. Although the pigs used in experiment 755 were older, it was considered appropriate to sacrifice the pigs 17 hours after injection.

There were similar trends in the levels of radioactive organic sulfur after decalcification and in total S^{35} in the region of the costochondral junction as seen, respectively, by radioautography and chemical analysis (figs. 3 and 4). Again the sodium versenate at pH 7.5 removed more of the organic sul-

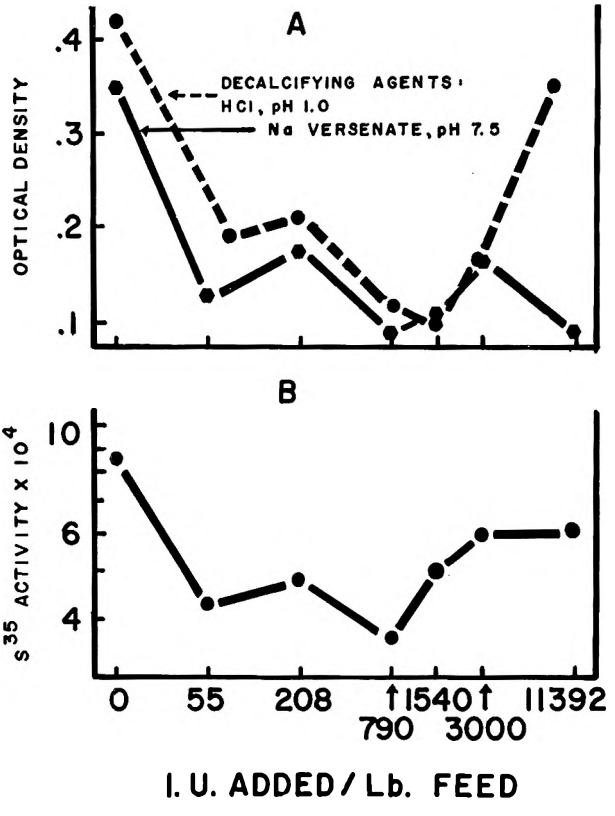


Fig. 3 Experiment 755. A, Effect of level of dietary vitamin A on the organic S^{35} activity of 4th rib costochondral junction as measured by the optical density of radioautographs prepared from 7 micron longitudinal sections of the tissue decalcified by two different reagents. B, Influence of vitamin A dosage level on the total S^{35} activity of the 5th rib costochondral junction as measured by the activity in counts per minute per gram of fresh tissue corrected for decay, background and self absorption.

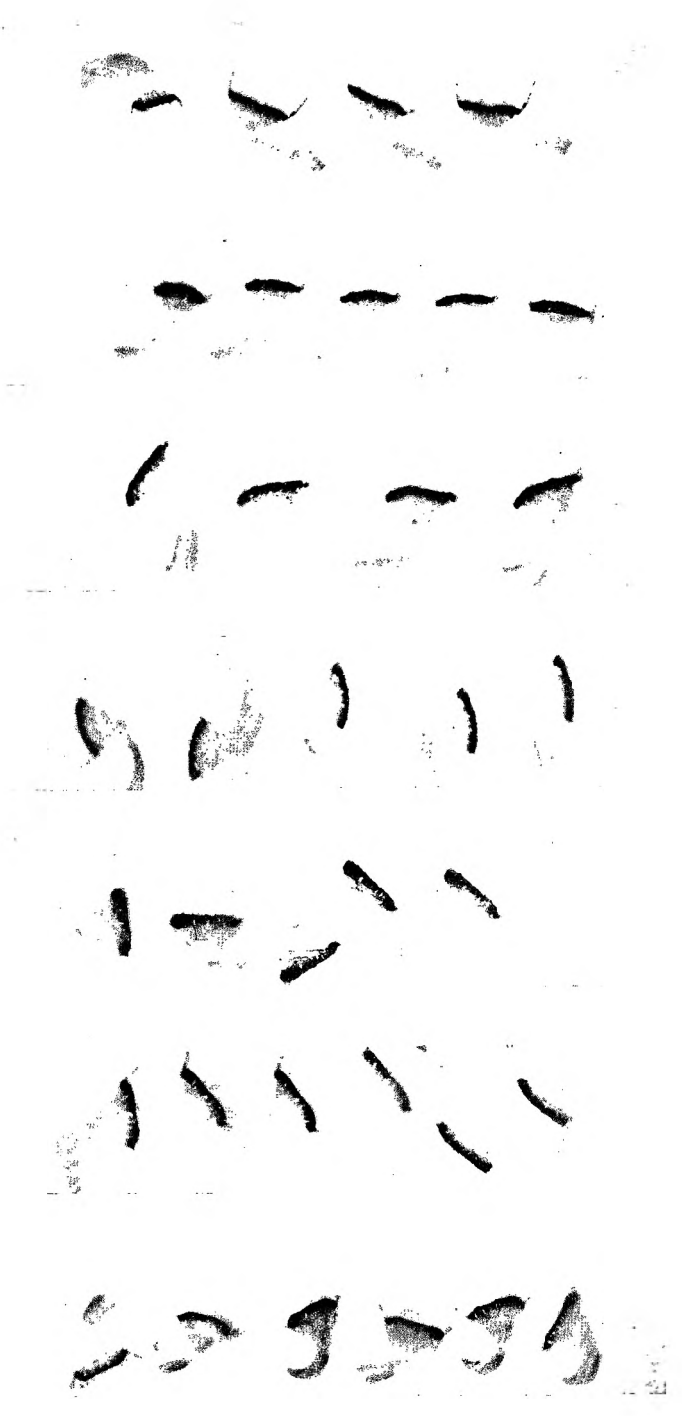


Fig. 4 Experiment 755. Samples at random of radioautographs from costochondral junctions decalcified in HCl. The sections were exposed to the film for 14 days. The letters A through G refer to dietary vitamin A levels 0 through 11,393 I.U. per lb. of feed.

fate than did the HCl. A correlation (r) of 0.935 was observed for the activities of this region as measured by chemical analysis and the average optical densities of the autographs for both decalcification procedures. The general trend (table 2) was for the activities to decrease then to increase with increasing dietary vitamin A. The upturn in the activity curve at higher levels of the vitamin corresponded to the significant growth depressions previously noted (Frape et al., '59). It required only 55 units of vitamin A per pound of diet to bring

TABLE 2

S^{35} activity in tissues from pigs on various dietary vitamin A levels

Experiment 755

VIT. A ADDED PER LB. OF FEED	5TH RIB COSTOCHONDRAL JUNCTION [1]	EAR CARTILAGE [2]	LUNG [1]	BLOOD PLASMA [1]
I.U.		*counts/min./gm fresh tissue* [3]		
0	87581	21213	7053	10951
55	42622	26764	3442	2268
208	48601	5902	4033	5163
790	32554	10811	1731	1655
1540	46789	12812	1346	1158
3000	54014	17082	1761	1653
11,393	53221	22238	2087	1710

[1] Values are average of two samples.

[2] Values are averages of three samples.

[3] Counting rates are corrected for background, decay and extrapolated to zero thickness of precipitate.

about a considerable reduction in the activity of the rib junction, lung tissue and blood plasma.

The trend toward reduced activity with the addition of vitamin A is the reverse of that reported by Dziewiatkowski ('54), where vitamin A administered to depleted rats brought about an increase in S^{35} accumulation in the epiphyseal plate region despite a decrease in the concentration of inorganic sulfate in the serum. In the present study, partial correlation analysis indicated that when growth rate is held constant, S^{35} activity of the ribs increases with vitamin A level in the diet, while the S^{35} activity of the plasma decreases. This reversal suggests

that the rats employed by Dziewiatkowski ('54), which were all initially depleted, probably were not growing appreciably when injected with vitamin A and S^{35}. Further, these rats were about three times the physiological age of the pigs in experiment 755.

The daily weight gain, blood plasma and liver vitamin A, and cerebrospinal fluid pressure data for these pigs are shown in table 3. The spinal fluid pressures followed a trend much like that for the S^{35} accumulation in the rib junction; each of these measurements decreased as the dietary vitamin A level

TABLE 3

Effect of level of dietary vitamin A on the daily weight gains, spinal pressures, blood plasma and liver vitamin A of pigs

Experiment 755

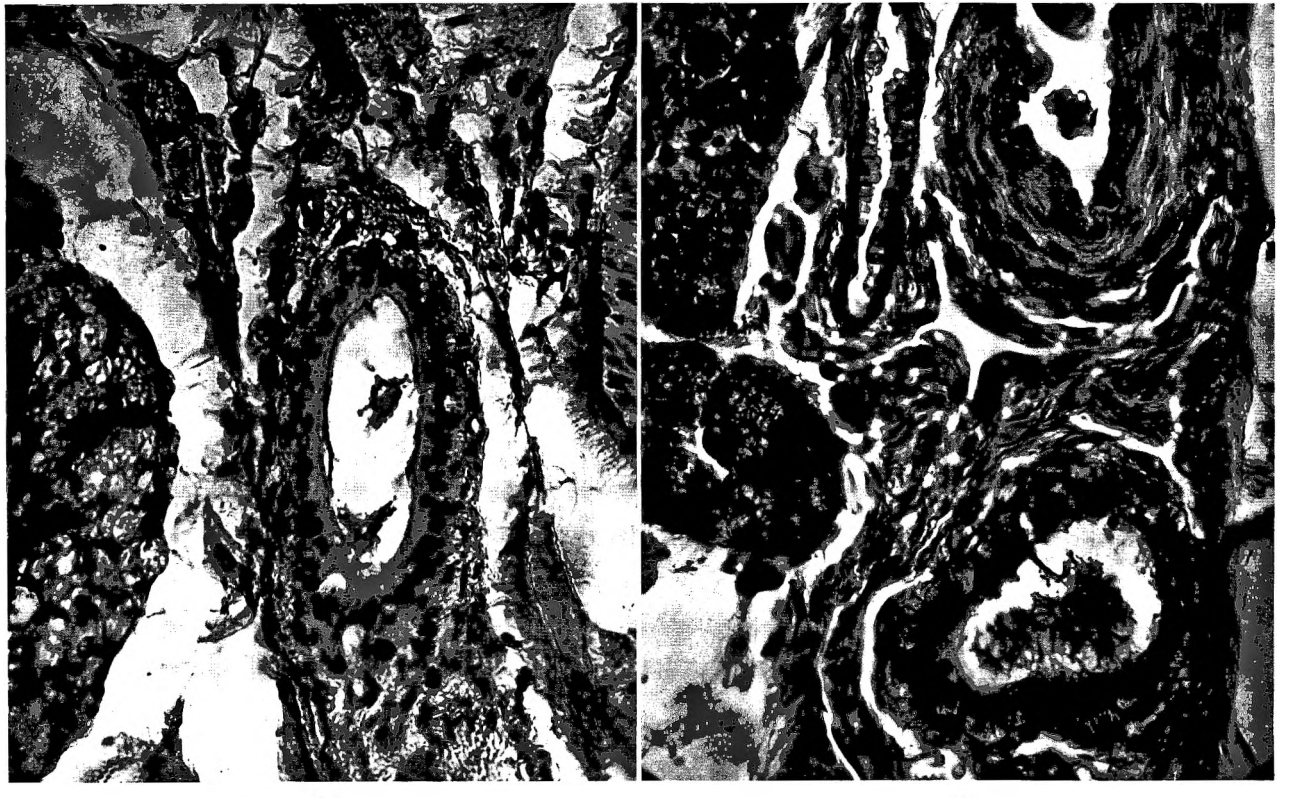
VIT. A ADDED PER LB. OF FEED	GAIN [1]	SPINAL FLUID PRESSURE	VITAMIN A LEVELS	
			Liver	Blood plasma
I.U.	*lb.*	*mm Saline*	*μg/gm*	*μg/100 ml*
0	0.18	320	0.0	2.7
55	1.68	230	0.3	3.3
208	0.82	105	0.1	9.2
790	1.68	85	2.9	27.2
1540	1.78	116	7.7	—
3000	1.42	118	24.4	31.1
11,393	1.34	58	123.0	33.0

[1] Average daily gain between 7 and 8.5 weeks of age.

increased to 790 I.U./lb. of feed. These observations support the hypothesis of Mellanby ('44) that an elevated spinal fluid pressure in vitamin A deficiency is brought about principally by bone growth abnormalities.

The depth of color of the thyroid glands, as measured by optical density readings of the photographs, increased as the level of vitamin A in the diet increased up to 1540 I.U./lb. of feed. At the two highest levels of vitamin A there was a decrease in redness of these glands.

The longitudinal sections of the costochondral junction showed no obvious abnormalities such as those observed by Jonsson et al. ('44) in dairy calves. This would support the



A B

Fig. 5 Experiment 755. A, Parotid salivary gland 6-micron section stained with hematoxylin and eosin. Normal gland from pig receiving adequate vitamin A. At left secretory alveolar tissue. Central interlobular connective tissue with normal interlobular duct lined with columnar epithelium. B, Parotid salivary gland 8-micron section stained with hematoxylin and eosin. Pathological condition in pig on basal diet. At left normal alveolar tissue. Remainder of photograph shows interlobular connective tissue containing a normal venule top, and a normal interlobular duct bottom right. The interlobular duct top right is lined with irregularly built-up metaplastic squamous keratinized epithelium associated with vitamin A deficiency.

work of Mellanby ('44), who was unable to find significant structural effects of vitamin A insufficiency on endochondral bone growth. Sections of the parotid salivary glands only rarely showed squamous metaplasia of the interlobular ducts (fig. 5) in the pig receiving the basal diet. This observation may add to the evidence for species variations in vitamin A deficiency symptoms, since Jungherr et al. ('50) found this gland to be the only organ in the ox that lends itself to specific morphologic diagnosis of vitamin A deficiency.

SUMMARY

Two experiments were employed in studying the relationship between vitamin A and S^{35} metabolism in pigs. The first showed that maximum accumulation of S^{35} in blood and lung tissues occurred 9 hours or less following intraperitoneal injection of $Na_2S^{35}O_4$; about 17 hours were required for maxima in ear cartilage and the costochondral junctions. In the second experiment, pigs maintained at various levels of dietary vitamin A were sacrificed 17 hours after intraperitoneal injection of $Na_2S^{35}O_4$. An extreme deficiency in vitamin A effected a high concentration of S^{35} in all tissues, especially in the rib junction. The addition of dietary vitamin A considerably reduced this activity; minimum activity occurred at the level of 790 I.U./lb. of feed. Higher levels of the vitamin A (up to 11,393 I.U./lb. of feed) brought about some return to higher specific activities in the tissues investigated. The relationships between S^{35} accumulation, growth and cerebrospinal fluid pressure suggest that the sulfur metabolism of the growing regions of bones is intimately concerned with bone growth and the pathological spinal pressures in vitamin A deficiency. The appearance of metaplasia in the interlobular ducts of the parotid gland would seem to be less frequent in the pig than that reported for the bovine animal. Color of the thyroid glands was influenced by level of vitamin A intake. Pigs receiving the levels of vitamin A near the optimum for maximum weight gain possessed the darkest colored glands.

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EGG PROTEIN AS A SOURCE OF THE ESSENTIAL AMINO ACIDS

REQUIREMENT FOR NITROGEN BALANCE IN YOUNG ADULTS STUDIED AT TWO LEVELS OF NITROGEN INTAKE [1]

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Sherman et al. ('20), Sumner et al. ('38), Bricker et al. ('45) and Hegsted et al. ('46) are among the investigators who have used the nitrogen balance technique with adult subjects to ascertain the minimum requirements for proteins such as those in egg, milk, white flour, soy flour, and meat when a single food constitutes the chief source of dietary nitrogen. In some instances it has been shown that supplementation of the food with an essential amino acid will reduce the amount required for nitrogen equilibrium. A notable example is the supplementation of white flour with lysine (Bricker et al., '45). However, it seems possible that for some food proteins, total nitrogen, rather than an essential amino acid, may become the limiting factor as the amounts fed are decreased. The question as to whether essential or total nitrogen is limiting might well be raised in regard to egg protein, the protein of highest biological value (Sumner and Murlin, '38). It is of interest that the minimum requirement for egg protein nitro-

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gen as determined by Sumner and co-workers ('38) closely parallels the minimum requirement for total nitrogen reported by Rose and Wixom ('55). Their value of 3.5 gm of total nitrogen per day was obtained in an experiment with young men ingesting an adequate and constant amount of an essential amino acid mixture and varying quantities of non-essential nitrogen.

In the present study an attempt was made to evaluate egg protein solely as a source of essential amino acids by determining the minimum amount of egg necessary to maintain nitrogen balance in the presence of an adequate total nitrogen intake. In a series of experiments, the amount of egg in the diet was reduced in stepwise fashion, while the total nitrogen was kept at a constant level by adding non-essential nitrogen in the form of glycine and diammonium citrate.

This study was also carried out to obtain evidence as to whether essential amino acid requirements for nitrogen equilibrium are influenced by the amount of total nitrogen in the diet. For this purpose, the amount of egg as the source of essential amino acids required to maintain nitrogen equilibrium was determined at two levels of total nitrogen intake, namely 6.5 gm N and 13 gm N per day. There is some evidence that for growth in experimental animals, the requirements of individual amino acids increase with increasing protein intake (Sauberlich et al., '53; Allison, '55).

EXPERIMENTAL

The experimental plan was to determine nitrogen balance in subjects fed varying amounts of whole egg in successive dietary periods with a constant total nitrogen intake of either 6.5 or 13 gm per day. The subjects were young men and women students.[2] A total of 11 subjects participated and 6 of these were studied at both nitrogen intake levels. They were judged to be in good health on the basis of a thorough medical examination. Their ages, weight and caloric intakes during the

[2] University of California, Los Angeles.

study are recorded in table 1. They maintained their usual activities while on the diet and ate two of their three daily meals under supervision in the diet kitchen.

The subjects were placed first on a controlled diet of ordinary foods containing 6.5 gm of nitrogen. This regimen was

TABLE 1

Sex, age, weight and caloric intake of each subject

SUBJECT NO.	NITROGEN IN DIET	AGE	WEIGHT		ENERGY VALUE IN DIET
			Initial	Final	
	gm per day	*yrs.*	*kg*	*kg*	*Cal. per day*
Men					
1	7.0	19	63.9	64.7	3600–3700
	13.0		64.7	65.2	3700
2	6.5	20	73.5	74.7	3500–3900
3	6.5	21	60.0	60.8	3200–3500
	13.0		62.4	64.1	3500
4	13.0	24	70.5	70.2	3500
5	13.0	20	59.8	60.7	3600–3700
Women					
6	6.5	20	50.9	52.3	2600–2700
	13.0		52.8	52.7	2700–2800
7	6.5	18	50.8	52.0	2600–2700
	13.0		51.3	52.8	2800
8	6.5	21	60.8	61.8	2600–2800
	13.0		61.3	61.7	2800
9	6.5	21	60.9	62.2	2600–3000
	13.0		61.1	62.6	3000
10	13.0	30	51.6	52.5	2600–2900
11	13.0	21	54.3	56.6	2600–2800

continued for 7 days or until nitrogen equilibrium was established. An isonitrogenous semi-synthetic diet was then substituted consisting of some low-protein fruits and vegetables, centrifuged butter, sucrose and cornstarch with mineral and vitamin supplements (Swendseid et al., '56). The caloric intake varied from 45 to 50 Cal. per kg of body weight, with the men being at the upper limit. The essential amino acids were

provided by whole egg which was hard-cooked and put twice through a Foley sieve. The amount of whole egg fed per day was equally distributed over the three meals and was varied in successive dietary periods of 7 or 8 days to provide information as to the minimum amount required for nitrogen equilibrium.

TABLE 2

Sources of dietary nitrogen

COMPONENT	6.5 GM N INTAKE		13 GM N INTAKE	
	Amt.	N content	Amt.	N content
	gm	*gm*	*gm*	*gm*
Fruits and vegetables	430	0.5	430	0.5
Glycine [1]	21.4	4.0	34	6.4
Diammonium citrate [1]			34	4.1
Essential amino acids in egg [1,2]	6.45	0.75	6.45	0.75
Non-essential amino acids in eggs [1,2]	8.80	1.25	8.80	1.25

[1] The amounts of these substances vary depending on the amount of whole egg in the diet. The values given in this table are for periods where 100 gm of whole egg are consumed.

[2] Calculated from Orr and Watt, '57. Cystine and tyrosine are included with the essential amino acids.

Although the amount of whole egg and therefore of essential amino acids varied, the total nitrogen of the diet was kept constant by adjusting the supplemental nitrogen, which was glycine, at 6.5 gm N and a mixture of glycine and diammonium citrate (DAC) at 13 gm N per day. Table 2 shows the distribution of dietary nitrogen at the two levels of intake.

Nitrogen analyses were carried out on food samples, daily urine collections and 5- or 10-day fecal pools. In computing the average daily fecal nitrogen, the values obtained for the entire experimental period, with the exception of the natural food diet, were used. Determinations were made according to the boric acid modification of the Kjeldahl procedure (Scales and Harrison, '20).

RESULTS AND DISCUSSION

Table 3 shows average daily nitrogen balance values for the various intakes of egg when the total dietary nitrogen was

6.5 gm per day. Of the three men, subject 1 stored nitrogen on 80 gm of egg, subject 3 stored nitrogen on 100 gm and subject 2 approached nitrogen equilibrium on 120 gm. All of the 4 women, subjects 6, 7, 8, and 9 stored nitrogen at a 100 gm intake of egg and showed nitrogen loss at an 80 gm intake.

By the Rose ('49) definition of adequacy, nitrogen equilibrium or nitrogen storage, 5 of 7 subjects obtained adequate amounts of essential amino acids from 100 gm of egg and one additional subject required only 80 gm. By the Leverton "zone

TABLE 3

Nitrogen balance on varying amounts of egg when the total nitrogen intake is 6.5 gm per day

Average daily nitrogen balance values[1] on indicated intake of whole egg

SUBJECT NO.	WHOLE EGG, GM/DAY			
	120	100	80	65
	gm N	*gm N*	*gm N*	*gm N*
Men				
1			+ 0.30	— 0.84
2	— 0.01	— 0.49	— 0.61	
3		+ 0.14	— 0.25	
Women				
6		+ 0.24	— 0.10	— 0.38
7		+ 0.21	— 0.33	— 0.73
8		+ 0.12	— 0.12	— 0.44
9		+ 0.03	— 0.71	

[1] Values recorded are for the last 4 days of a 6–8 day dietary period.

of equilibrium" (Leverton et al., '56), 5 of 7 subjects were supplied with adequate amounts of essential amino acids from 80 gm of egg. By both criteria, 65 gm of egg proved to be inadequate for all subjects.

It can be calculated from the protein content of egg (Block and Weiss, '56) that the egg protein requirement for these 7 subjects in an experiment where adequate amounts of non-essential nitrogen were supplied ranged from 10 to 15 gm per day. This is a requirement below the value of approximately 20 gm obtained by Sumner and Murlin ('38) when they fed

egg as the chief dietary nitrogen source. These data indicate, therefore, that in dietary situations where egg is the sole protein, source, the total nitrogen content rather than an essential amino acid becomes the limiting factor in nitrogen balance experiments.

The range of egg protein requirement for the 7 subjects represents the equivalent of from 0.6 to 0.9 gm N in the form of essential amino acids (table 2). (The additional contribution from low-protein fruits and vegetables is less than 0.1 gm N.) These values can be compared with two experiments where essential amino acid mixtures were administered in ratios based on their individual requirements. Rose and Wixom ('55) fed young men essential amino acids at twice their highest required level in an amount of 1.42 gm N. Swendseid and Dunn ('56) maintained young women in nitrogen balance on the calculated equivalent of 0.46 gm N given as essential amino acids. It is evident that more experiments must be performed before conclusions can be drawn as to the relative efficiencies of different amino acid patterns and the availability of amino acids in food protein.

There is also the question of identifying the limiting essential amino acid in egg protein. From consideration of analytical data on egg protein composition (Orr and Watt, '57) and tentative suggested requirements of essential amino acids for young men and women (Rose, '49; FAO Nutritional Studies, '57), methionine was selected for preliminary investigation. In three subjects a supplement of 300 mg of methionine daily at the 80 gm egg intake level did not produce nitrogen storage although in two subjects the amount of nitrogen loss was reduced (from — 0.25 to 0.12 and from — 0.71 to — 0.30 gm per day). The third subject showed no change in nitrogen balance.

Table 4 gives data showing the effect of increasing the total supplemental nitrogen on the requirement for egg as a source of essential amino acids. The 13 gm nitrogen intake level is higher than those previously reported for semi-synthetic diets fed to young adults (Rose, '49; Leverton et al., '56) and is

twice the amount used in the first part of the study. This high level was chosen as a means of accentuating any differences that might occur in amino acid requirements. The supplemental nitrogen used was a mixture of glycine and diammonium citrate (table 2). It was found that when glycine alone was fed in the large amount required to bring the nitrogen

TABLE 4

Nitrogen balance on varying amounts of egg when the total nitrogen intake is 13 gm per day

Average daily nitrogen balance values [1] on indicated intake of whole egg

SUBJECT NO.	WHOLE EGG, GM/DAY				
	200	150	120	100	80
	gm N	*gm N*	*gm N*	*gm N*	*gm N*
Men					
1 [2]		+ 0.42	− 0.34		
3 [2]				+ 0.42	+ 0.45
4	− 0.06	− 1.14	− 0.83		
5		+ 0.77	+ 0.29	+ 0.21	+ 0.91
Women					
6 [2]		+ 0.59	− 0.30		
7 [2]				+ 0.89	− 0.26
8 [2]				+ 0.13	− 0.26
9 [2]				+ 0.81	− 0.16
10		− 0.03	− 0.24	− 0.40	
11		+ 0.64	+ 0.62	+ 0.70	+ 0.01

[1] Values recorded are for the last 4 days of a 6 to 8 day dietary period.

[2] These subjects also participated in the 6.5 gm nitrogen intake study.

intake to 13 gm, the urinary excretion of this amino acid was greatly increased.[3]

Of 10 subjects on the 13 gm nitrogen intake, 3 (subjects 3, 5 and 11) stored nitrogen on 80 gm of egg, three (subjects 7, 8 and 9) required 100 gm, and three (subjects 1, 10 and 6) either stored nitrogen or approached equilibrium on 150 gm. Subject 4 approached nitrogen balance only when receiving 200 gm of egg. It appears that there is a greater variation in the requirements for egg as a source of essential

[3] Feeley et al., unpublished results.

amino acids at this higher nitrogen level. However, for a given individual, there is no correlation between requirements at the two levels of nitrogen intake. Of the 6 subjects studied on both levels, two (subjects 1 and 6) showed definitely increased requirements on the higher nitrogen intake while the requirement for one (subject 3) was slightly decreased and for three (subjects 7, 8 and 9) remained the same. No evidence could be obtained, therefore, under conditions of this experiment for a consistent relationship between amino acid requirements and nitrogen intake. The results might be explained on the basis of individual differences in the utilization of non-essential nitrogen at the two intake levels. They suggest that further experiments should be carried out at other nitrogen levels and with different sources of supplemental nitrogen. These results with young adults differ from a study reported with older men (Tuttle et al., '58) where a definite increase in essential amino acid requirements occurred with increasing nitrogen intake in 7 of 8 subjects. For the older men, the essential amino acids were given in the form of a synthetic mixture of the L-isomers patterned after egg protein; otherwise conditions in the two studies were similar.

SUMMARY

Young men and women subjects were fed varying amounts of egg as a source of the essential amino acids, with the total nitrogen in the diet being held constant at either 6.5 gm or 13 gm per day by the addition of non-essential nitrogen in the form of glycine and diammonium citrate. In 6 of 7 subjects, nitrogen equilibrium was attained when 100 gm of egg supplied the essential amino acids at 6.5 gm total nitrogen intake. The range of requirements was from 80 to 120 gm of egg, (10 to 15 gm of egg protein). At a 13 gm total nitrogen intake, the range of requirements for nitrogen equilibrium was from 80 to 200 gm egg. The 6 subjects who were studied at both levels of nitrogen intake did not show a consistent variation in the requirement for egg and thus for essential amino acids when the dietary nitrogen was increased.

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THE DOMESTIC CAT AS A LABORATORY ANIMAL FOR EXPERIMENTAL NUTRITION STUDIES

VII. PYRIDOXINE DEFICIENCY

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Pyridoxine deficiency has been described for various avian and mammalian species. The following deficiency signs have frequently been reported: growth depression, alterations of skin, fur or feathering, microcytic hypochromic anemia with high serum iron, abnormality of tryptophan metabolism with xanthurenic acid excretion in urine, convulsive seizures and histologic lesions (kidneys, adrenals, heart, central and peripheral nervous system and arteries).

In this paper are presented data demonstrating that the domestic cat requires pyridoxine for normal growth and blood picture. Convulsive seizures, decreased xanthurenic acid excretion even after tryptophan load, pyridoxine levels in tissues, and kidney lesions are described. Data on blood and plasma volume, total body water, blood glucose, pyruvate

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and lactate, plasma sodium, potassium, calcium and inorganic phosphorus are also presented.

EXPERIMENTAL

The detailed techniques used in working with cats have already been described (Carvalho da Silva, '50a, b). Cats three to 4 months old were placed in screen-bottom cages kept in a constant-temperature room (24 to 26°C); they received water and the following purified ration ad libitum: "vitamin free" casein,[5] 33; gelatin, 2; lard, 5; peanut oil, 5; salt mixture IV (Phillips and Hart, '35), 5; sucrose to make 100%. When the amount of casein was raised to 45% or when tryptophan was added, an equivalent amount of sucrose was deducted. This diet was supplemented with vitamins administered separately. One milligram each of thiamine, riboflavin, vitamin K, and *p*-aminobenzoic acid; 4 mg of calcium pantothenate; 10 mg of niacin; 0.5 mg of folic acid; 0.01 mg of *d*-biotin; 30 mg of inositol and 300 mg of choline were dissolved in 1 ml of water, this mixture was emulsified with 2 ml of cod liver oil and the resulting product was given by mouth three times a week. Once a week, 15 mg of α-tocopherol were added. The control animals received the same diet with the addition of 1 mg of pyridoxine to the vitamin supplement. It has been demonstrated that this ration promotes a satisfactory growth rate and blood picture (Carvalho da Silva, '50b), although it is probably low in choline (Carvalho da Silva et al., '59).

The techniques used for hematological studies, determination of plasma iron, plasma and blood volumes, and urine collection, have been previously described (Carvalho da Silva et al., '55). Pyridoxine was assayed with *S. carlsbergensis* (Atkin et al., '43); adequate amounts of tissues were ground with washed sand and autoclaved for one hour at 15 pounds of pressure, with 0.5 N hydrochloric acid. Xanthurenic acid was determined by the method of Rosen et al. ('51); the

[5] "Acid extracted" (Cannon et al., '45) or "Labco vitamin-free."

approximate concentration in each sample of urine was evaluated by a preliminary trial, and final determinations were made on amounts of urine containing approximately 150 μg of xanthurenic acid. To each sample, 150 μg of pure xanthurenic acid were added as an internal standard. The cages used for collection of the urine for these determinations were coated with paraffin, in order to minimize the contact of urine with iron.

Body water was determined by the antipyrine method (Brodie, '51), adapted as follows (Carvalho da Silva and Pontes, '57): purified antipyrine, 25 mg/ml in 5% glucose solution, was injected by peritoneal route in the proportion of 2 ml of solution per kilogram of body weight;[6] the exact amount injected was calculated by weighing the syringe with the needle before and after the injection, and correcting for the specific gravity of the solution, as evaluated with a picnometer. Three-milliliter blood samples were collected from the saphenous veins with heparinized syringes, before and 60, 90, 120 and 150 minutes after the injection. The blood was centrifuged and 1 ml of plasma diluted with 3 ml of distilled water and treated as described by Brodie ('51) to determine the concentration of antipyrine as 4-nitroso-antipyrine. The theoretical concentration at zero time was divided by 0.94 to correct for the plasma protein (Carvalho da Silva et al., '58).

The following determinations were carried out on total blood collected from the saphenous vein with heparin as the anticoagulant: glucose (Folin and Svedberg, '30); pyruvic acid (Bueding and Wortis, '40); lactic acid (Barker and Summerson, '41). Sodium and potassium (Hald, '51), calcium (Halverson and Bergeim, '17) and inorganic phosphorus (Fisk and Subbarow, '25) were determined on plasma obtained from blood collected by heart puncture under anesthesia.[7]

[6] Peritoneal route was selected because intravenous administration of antipyrine to cats produces voluminous salivary secretion containing antipyrine.

[7] Nembutal was used.

The tissues for pyridoxine determinations and histologic sections were taken from animals which were deeply anesthesized.[7]

RESULTS

Growth. The average growth rates for pyridoxine-deficient and control animals (purified ration plus pyridoxine), are presented in figure 1. The weight gains of the deficient cats were interrupted by the end of the third week for the males and by the end of the second week for the females; however, individual variations were marked; some animals stopped growing after a few days on the experiment, while others gained weight until other signs of deficiency such as anemia and convulsions, were present. Although high protein levels aggravate pyridoxine deficiency (Sherman, '54), no differences were observed among cats receiving either 33 or 45% of casein. Levels below 33% were not tried in view of the high protein requirement by this animal species (Carvalho da Silva et al., '58).

Convulsive seizures. Thirteen out of 28 cats exhibited convulsive seizures after an average of 87 (37 to 127) days on experiment. The seizures started suddenly, lasting for 5 to 15 seconds as a generalized convulsion, with mild salivation and dilatation of the pupils; finally the muscles relaxed and the animals appeared to be waking. Between the convulsions they moved and ate normally; it was even common to observe weight gains for a few days after the first convulsions. After one or two weeks, the seizures occurred more frequently and the animals became first depressed and then excitable. Attempts to induce the convulsions with flashes of light in a dark room, sudden noises, or by dropping the animals to the floor, failed even when tried on cats exhibiting several attacks daily; intravenous administration of 3 to 5 ml of 50% glucose solution gave appreciable relief for a few days.

Hematological data. The blood picture was studied in a group of 9 animals before and after the pyridoxine-deficient

[7] See footnote 7, page 215.

diet had been fed for 90 days. The results (table 1, group A) indicate that pyridoxine deficiency produces a mild microcytic hypochromic anemia. The reductions in the values for hemoglobin, red cells, mean corpuscular volume, mean corpuscular hemoglobin were significant by the "t" test with variates paired (Bernstein and Weatherall, '52). The average iron content of serum was 185 μg (123 to 320) per 100 ml for 5 pyridoxine-deficient cats and 78.3 μg (75 to 90) per 100 ml

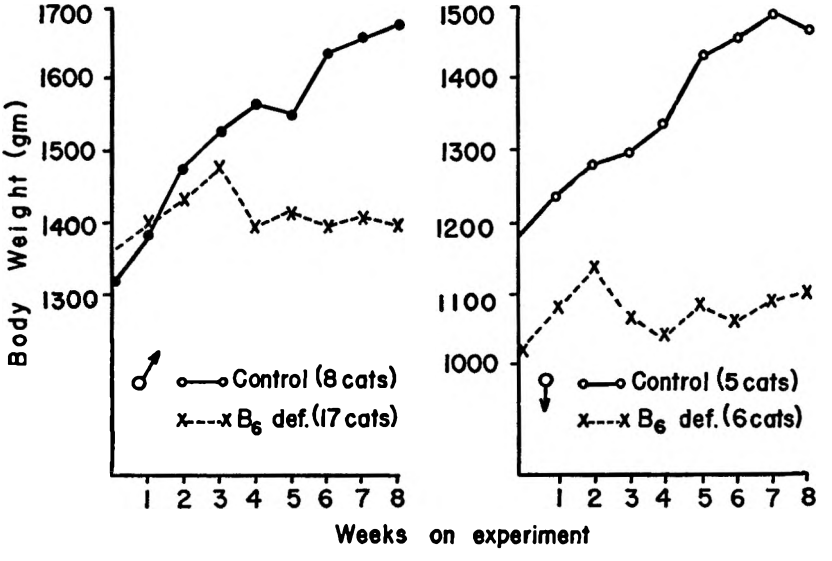

Fig. 1 Growth rate of male and female cats on control and pyridoxine-deficient diets.

for 6 controls. Another group of 5 animals was used to study the influence of folic acid restriction on the anemia produced by the pyridoxine-deficient diet. After a preliminary period of 60 days in which the purified diet without folic acid and without sulfa drugs was fed (Carvalho da Silva et al., '55), blood counts were performed and the animals were maintained for 90 days on the same diet but without pyridoxine and folic acid. A microcytic hypochromic anemia of the same degree as in group A, table 1, was observed, but the micro-

cytosis was less pronounced. The values for mean corpuscular volume before pyridoxine deficiency, were 43.8 μ^3 and after pyridoxine deficiency, 40.0 μ^3 (difference statistically significant, by the "t" test, with variates paired).

Response to treatment. Pyridoxine-deficient cats resumed growth and attained their normal weight values for age and sex when treated with 1 to 10 mg of pyridoxine daily. Although no satisfactory information on minimal dosage is available, one cat failed to respond to 0.25 mg of pyridoxine given daily for 6 days, but gave an immediate response to 1 mg daily; good recovery was obtained on another animal with 0.5 mg daily. The hematologic recovery was studied in a group of 6 cats with pyridoxine-deficiency anemia (table 1, group B); after one month on treatment with 1 to 10 mg of pyridoxine daily, the hematological values were equivalent to those observed during the control period.

Pyridoxine levels in tissues. The pyridoxine levels in liver, kidneys, skeletal muscle and brain were studied in 5 control and 5 deficient cats (table 2). The results indicate a reduction of approximately 50% of the vitamin stores in these animals as a result of the deficiency.

Urinary excretion of xanthurenic acid. The xanthurenic acid excretion was measured in controls and in pyridoxine-deficient cats with and without the addition of tryptophan to the diet. For comparison, simultaneous observations were made on Wistar albino male rats placed on experiment at the age of 30 days and receiving the same rations supplied to the cats. The results are presented in table 3. The cats were considered deficient when exhibiting convulsive seizures; the rats were considered pyridoxine deficient after 60 days on the deficient diet; by this time, they exhibited weight loss, unkempt fur and a mild dermatitis on the paws. Urine samples of each cat and of groups of three or 4 rats were collected during three to 5 successive days; the determinations were made on the pooled samples of each cat and of each group of rats. The food intake was measured during the collection period, in order to calculate the tryptophan ingested.

TABLE 1

Influence of pyridoxine deficiency on the hematologic values of the cat [1]

GROUP	NO. OF ANIMALS	SAMPLING PERIOD	HEMOGLOBIN	RED CELLS	MEAN CELL VOLUME	MEAN CORPUSCULAR HEMOGLOBIN
			gm%	*mm³ × 10⁶*	*μ³*	*μg*
A	9	Before the experiment	16.4	7.54	43.8	13.8
		Pyridoxine deficiency	7.0	6.49	34.9	10.5
			$p < 0.01$	$p < 0.05$	$p < 0.01$	$p < 0.01$
B	6	Before the experiment	11.5	8.63	43.1	13.4
		Pyridoxine deficiency	6.9	6.41	35.7	10.5
		After treatment with pyridoxine [2]	10.9	9.01	41.3	12.4
			$p > 0.1$ [3]	$p > 0.5$ [3]	$p > 0.5$ [3]	$p > 0.1$ [3]

[1] p (probability of chance difference), was calculated by the "t test" for variates paired.
[2] With 1 to 10 mg of pyridoxine daily.
[3] When control and after treatment periods were compared.

TABLE 2

Pyridoxine levels in the tissues of control and deficient cats

Averages and standard errors for groups of 5 animals

GROUP	PYRIDOXINE (μG/GM OF WET TISSUE)			
	Liver	Kidney	Brain	Skeletal muscle
Controls (purified ration with pyridoxine)	4.5 ± 0.59	4.1 ± 0.30	1.8 ± 0.016	4.0 ± 0.23
Pyridoxine-deficient	2.1 ± 0.19	1.9 ± 0.15	1.0 ± 0.036	2.2 ± 0.30

The tryptophan content of casein was calculated as 1.2% (Hawk et al., '54). It is evident from table 3 that the amount of tryptophan converted to xanthurenic acid by pyridoxine-deficient cats is very small, when compared to the results obtained on rats. The xanthurenic acid excretion observed in our pyridoxine-deficient rats was higher than values reported in the literature. This may be explained by the fact that we used 33% of casein and an experimental period of 60 days; also, Brown and Price ('56) demonstrated that too high values for urinary xanthurenic acid are obtained when the samples are assayed without adequate preliminary treatment.

Kidney lesions. Macroscopically, scarring and pitting were frequently observed in the kidneys of pyridoxine-deficient cats (fig. 2). These lesions were usually more evident if deficient animals were treated with pyridoxine until recovery, and subjected to a second deficiency.

Microscopically, deposits of crystalline material were observed in the lumen of the collecting and convoluted tubules, principally in the cortex but also in the medulla (fig. 3); occasionally, the tubules were completely obstructed. Some crystals were brown and without clear limits; others were basophilic in nature and exhibited sharp contours, suggesting calcified material. When observed with polarized light, they were birefringent (figs. 4 and 5). These crystalline formations did not melt at 330°C and were not affected by microincineration for 30 minutes at 700°C. Although their detailed composition was not established, the presence of calcium was demonstrated by the test of von Kossa and by the formation of acicular crystals after treatment with 2 N sulfuric acid (Lison, '53). The calcium was probably in the form of phosphate; carbonate could be excluded by the absence of gas bubbles after treatment with 2 N sulfuric acid.

The collecting and convoluted tubules were frequently dilated principally in areas containing crystals (fig. 4). The walls of the dilated tubules were lined with high cells presenting a pink cytoplasm and small nucleus, or cubic cells with

TABLE 3

Xanthurenic acid excretion in control and pyridoxine-deficient cats and rats

EXPERIMENTAL GROUP	NUMBER OF ANIMALS	TRYPTOPHAN INGESTED	XANTHURENIC ACID EXCRETED	
		mg/anim/day	*mg/anim/day*	*as % of ingested trypt.* [1]
Cats				
Controls	3	327	0.9	0.27
5% DL-tryptophan	3	5.200	5.2	0.10
Pyridoxine deficiency	4	112	1.1	1.01
Pyridoxine deficiency + 5% DL-tryptophan	4	969	3.6	0.38
Rats				
Controls	4	39	non-dosable	—
5% DL-tryptophan	8	355	4.7	1.30
Pyridoxine deficiency	11	25	8.1	32.40
Pyridoxine deficiency + 5% DL-tryptophan	12	156	53.2	34.10

[1] On a weight basis, since xanthurenic acid and tryptophan have practically the same molecular weight.

TABLE 4

Comparative data on control and pyridoxine deficient cats

	CONTROLS		PYRIDOXINE-DEFICIENT	
	Number of animals	Average and limits	Number of animals	Average and limits
Blood volume (ml/100 gm body wt.)	11	6.9 (5.3–7.8)	4	6.2 (5.6–6.8)
Plasma volume	11	3.9 (3.0 4.5)	4	3.8 (3.6–4.2)
Total body water	12	62.6 (54.3–74.2)	5	62.9 (60.4–67.2)
Blood glucose (mg/100 ml blood)	20	96.1 (58.0–137.0)	9	105.0 (76.0–125.0)
Blood pyruvate (mg/100 ml blood)	20	1.4 (0.8–1.8)	10	1.5 (1.1–2.5)
Blood lactate [1] (mg/100 ml blood)	21	22.8 (16.4 29.6)	10	33.9 (11.7–70.8)
Plasma sodium (meq/l)	7	154.1 (125.0–170.0)	4	150.5 (148.0–153.0)
Plasma potassium (meq/l)	6	4.1 (3.8–5.9)	4	4.6 (4.1–4.8)
Plasma calcium (mg/100 ml)	8	10.0 (9.2–11.0)	4	11.5 (10.0–12.5)
Plasma inorganic phosphate (mg/100 ml)	6 [2]	7.1 (6.2–7.5)	4	7.6 (6.8–8.3)

[1] Difference between controls and pyridoxine-deficient not statistically significant ($t = 0.573$).

[2] Cats 3 to 4 months old.

scanty basophilic cytoplasm and voluminous nucleus. In the lumen of the enlarged tubules, deposits of an amorphous pink material were frequently observed; tubular atrophy was also evident. The glomeruli, blood vessels and interstitial material were normal.

Deficient animals, treated with pyridoxine and subjected to a second pyridoxine deficiency showed identical lesions but with more conspicuous and diffuse tubular dilatation and atrophy, and mononuclear infiltration and fibrosis, giving a typical aspect of pyelonephritis. Extensive fibrosis including the cortex and the medulla were observed in some animals (fig. 7).

Deficient animals treated with pyridoxine, 1 to 10 mg daily for more than 30 days, showed also tubular dilatation with interstitial fibrosis and infiltration, but with small amounts of crystalline material (fig. 8).

Other experimental data — Blood, plasma and body water volumes, blood glucose, pyruvate and lactate, plasma sodium, potassium, calcium and inorganic phosphorous, were determined on control and pyridoxine deficient cats; no differences were observed (table 4).

DISCUSSION

The microcytic hypochromic anemia with high serum iron, observed in pyridoxine-deficient cats, is in accordance with the results obtained for ducks (Hegsted and Rao, '45), dogs (Fouts et al., '38; Street et al., '41; Fouts and Lepkovsky, '42; McKibbin et al., '42), pigs (Wintrobe et al., '42, '43), rhesus monkey (McCall et al., '46; Poppen et al., '52) and humans (Harris et al., '56). Although anemia has not been regularly observed in pyridoxine-deficient rats, impairment of blood regeneration is demonstrable after experimental hemorrhage (Kornberg et al., '42; Hawkins and Lechow, '52). In contrast to our results on cats, McCall and associates ('46) did not observe a complete hematologic recovery in pyridoxine-deficient monkeys after pyridoxine treatment. This dif-

ference may be related to the requirement for an "antianemia factor" by this last species (Smith and Elvehjem, '51).

Convulsive seizures have been reported in turkeys (Bird et al., '43), chicks (Jukes, '39), ducks (Hegsted and Rao, '45), rats (Lepkovsky et al., '42), rabbits (Hove and Herndon, '57), dogs (Street et al., '51), pigs (Wintrobe et al., '42, '43) and humans (György, '54). Johnson and associates ('50) observed convulsive seizures in calves, lasting as long as 8 minutes. Our observation that the seizures in pyridoxine-deficient cats are spontaneous but cannot be elicited, are in accordance with the results of Bird et al., ('43) on turkeys. However, Lepkovsky and coworkers ('42) were able to increase the susceptibility to epileptic fits by oral administration of 5 ml of water to pyridoxine-deficient rats.

Diamant and Guggenheim ('57) observed a reduction in water excretion after administration of saline to pyridoxine-deficient rats, but Schwartzman and Strauss ('49) were not able to detect signs of water retention in the Syrian hamster; Davenport and Davenport ('48) obtained normal values for water, sodium and potassium levels in the brain and plasma of deficient rats. Our results on plasma volume, total body water and concentration of sodium and potassium in plasma, are in accordance with these last reports.

The normal values for blood pyruvate and lactate in pyridoxine-deficient cats agree with the results of Davenport and Davenport ('48) for rats.

The low excretion of xanthurenic acid in pyridoxine-deficient cats, even when 5% of DL-tryptophan was added to their diet is in accordance with the observation that this animal species does not form niacin from tryptophan (Carvalho da Silva et al., '52). Also, according to Brown and Price ('56), the excretion of known metabolites of tryptophan by the cat amounts only to approximately 0.1% of the amnio acid ingested; however, in rats and dogs, as much as 20 to 40% of the ingested tryptophan is excreted as known metabolic products.

Our data on pyridoxine levels of the tissues agree with the results of Sheppard and McHenry, ('46), who worked with rats.

Kidney lesions have been described by Agnew ('51) in hooded rats subjected to pyridoxine deficiency. In short-term experiments, 50% of his animals had hematuria. Although the kidneys were macroscopically normal, abundant deposits of amorphous faintly eosinophilic material were present in the subcapsular spaces. According to this investigator, the earliest lesion in pyridoxine-deficient rats with hematuria appears to be one affecting the glomerular filter and this lesion may be responsible for the amorphous material found in the subcapsular spaces and for the red cells found in the urine. In long-term experiments, Agnew ('51) describes scarring and pitting, fibrosis, tubular dilatation, deposition of cretaceous material and pyelonephritis. In our studies with cats, no deposits of subcapsular material or other indications of primary glomerular lesions were found; although we did not look for microscopic hematuria, macroscopic bleeding was not observed.

The exact relationship between the histologic lesions and the birefringent crystalline deposits in cats, was not determined with certainty. Although these crystals were more abundant in areas with advanced tubular lesions, they were also present in areas showing only a mild degree of tubular dilatation. This suggests that some unidentified tubular disfunction or abnormality in the composition of the glomerular filtrate is responsible for their formation. Once formed, the crystals could interfere with the normal flow of the glomerular filtrate along the tubules and even obstruct them and aggravate the tubular lesions. The fact that in animals that had recovered from pyridoxine deficiency by pyridoxine treatment, only small amounts of crystals were present although histologic lesions were pronounced suggests that some mechanism is available for reabsorption or elimination of this material.

Arteriosclerotic lesions in various organs as described in pyridoxine-deficient monkeys by Rinehart and Greenberg ('49) were not found in our pyridoxine-deficient cats.

SUMMARY

Pyridoxine deficiency was obtained in growing cats. The signs observed were growth depression, microcytic hypochromic anemia with high serum iron, convulsive seizures and kidney lesions, represented by areas of tubular atrophy and tubular dilatation, fibrosis and intratubular deposition of birefringent crystalline material. The pyridoxine levels in tissues were reduced to approximately 50% of the control values. Xanthurenic acid excretion was very low, even after tryptophan load. Blood and plasma volume, total body water, blood glucose, pyruvic and lactic acid, plasma potassium, sodium, calcium and inorganic phosphorus were not affected. Satisfactory body weight and hematologic recoveries were obtained with pyridoxine treatment but the kidney lesions were not reversible.

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PLATE 1

EXPLANATION OF FIGURES

2 Kidney of a pyridoxine-deficient cat (first deficiency).

3 Microscopic aspect of the kidney of a cat at the end of the first pyridoxine deficiency. Extensive tubular dilatation. Crystalline deposits in the tubular lumen. Bouin, Hematoxylin-eosin. × 400.

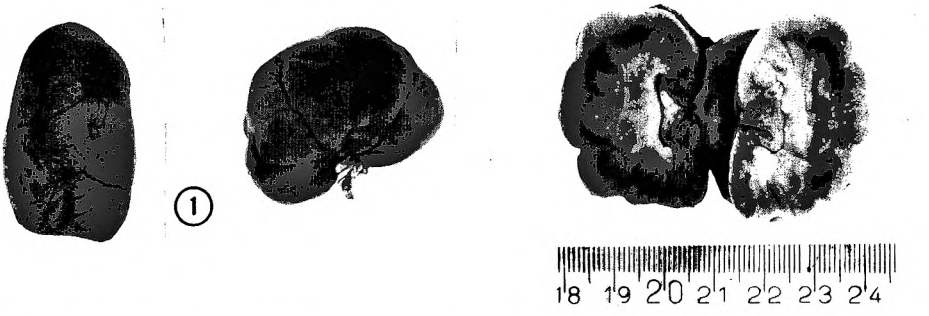
4 Area of the kidney of a pyridoxine deficient cat showing intratubular crystals.

5 The same area as figure 4 photographed with polarized light. Bouin, Hematoxylin-eosin. × 100.

6 Kidney of a cat subjected to a second pyridoxine deficiency. On the right side of the picture, interstitial fibrosis and infiltration are evident. Bouin, Mallory. × 400.

7 Extensive fibrosis of the cortical and medullar areas after a second pyridoxine deficiency. Bouin, Mallory. Small magnification.

8 Kidney of a cat subjected to pyridoxine deficiency and treated with pyridoxine (1 to 10 mg daily, during 30 days). On the left side the tubules are preserved but some of them show dilatation; on the right side fibrosis and tubular atrophy are evident. Helly, Hematoxylin-eosin. × 400.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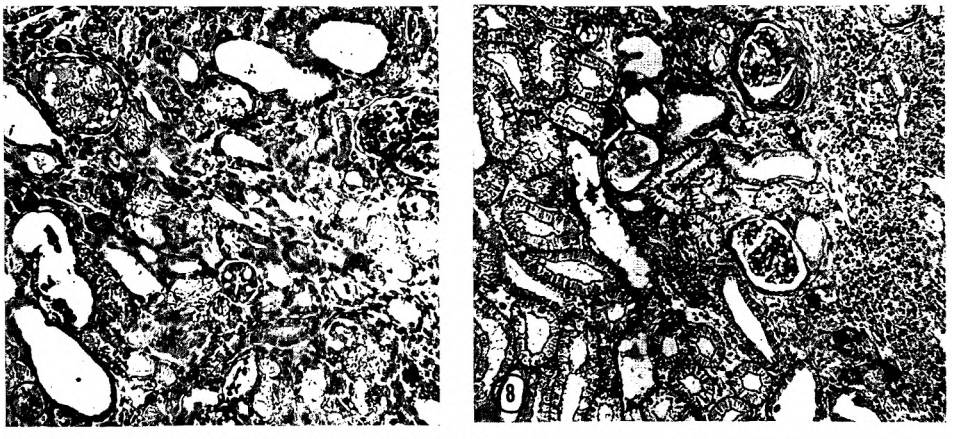
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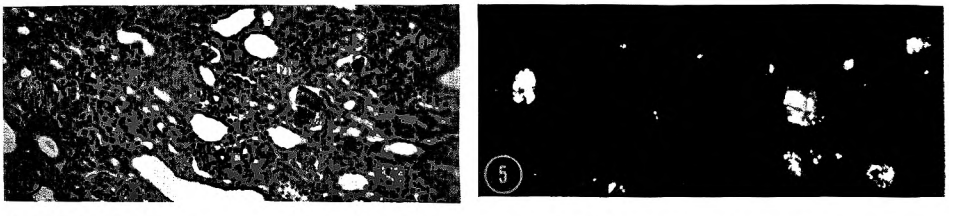
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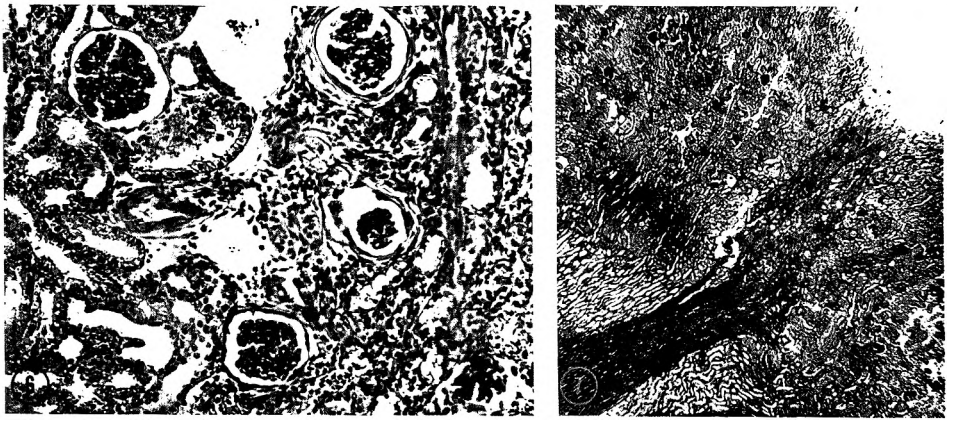
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THE EFFECTS OF DEFICIENCY OF B VITAMINS ON SALT TOXICITY IN THE RAT

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Although excessive intake of sodium has long been suspected as an important factor in the production of arterial hypertension in man (Allen, '20), little work has been reported on the effects of sodium chloride toxicity. Meneely et al. ('52) reported that rats fed diets containing from 2.8 to 9.8% of sodium chloride grew more slowly than those fed a similar diet containing 0.15% of sodium chloride. In further studies, Meneely et al. ('53) observed sustained arterial hypertension in rats fed excessive amounts of sodium chloride for 9 months, and noted a linear relationship between the level of sodium chloride in the diet and the systolic blood pressure. Meyer ('54, '55) studied interrelationships between sodium chloride and protein levels in diets for growing rats. He concluded that in animals fed ad libitum, the growth depression caused by the addition of excessive sodium chloride to the diet was independent of the dietary protein level. However, increasing the dietary protein level resulted in much greater renal hypertrophy in animals receiving 15.5% of sodium chloride, than in those receiving only 0.5% of sodium chloride.

Schaefer ('57) presented evidence, from preliminary experiments, that the toxicity of excessive sodium chloride is influenced by the intake of B vitamins and reported sea salt to be somewhat less toxic than an equivalent amount of sodium chloride. In the present studies, two experiments, one of 6 weeks and the other of 17 weeks duration were conducted to study the effects of deficiency of B vitamins on salt toxicity

in the rat. The results of both experiments show that excessive amounts of either sodium chloride or sea salt are much more toxic to rats fed diets deficient in B vitamins than to those animals receiving adequate amounts of B vitamins. Evidence was also obtained indicating that the cardiac hypertrophy brought about by administration of excessive sodium chloride to animals fed an otherwise adequate diet is partially overcome by addition of potassium chloride to the diet. Since the results of both experiments were in close agreement, only the results of the longer-term study are reported in detail herein.

EXPERIMENTAL

The basal 18% casein diet used in the experiment was similar to that of Sarett and Snipper ('54), except that sucrose was used as the carbohydrate, and certain changes were made in the amounts of B vitamins added to the diet. Twelve comparable groups of 10 male weanling rats each (McCollum-Wisconsin strain) were selected on the basis of litter origin and body weight. Six of the groups received the basal diet alone or supplemented with 3 or 6% of sodium chloride, 4 or 8% of sea salt (containing approximately 75% of sodium chloride) or 6% of sodium chloride and 2% of potassium chloride. These groups received the following amounts of B vitamins per 100 gm of diet: thiamine hydrochloride, 0.25 mg; riboflavin, 0.5 mg; niacinamide, 5.0 mg; calcium pantothenate, 2.0 mg; pyridoxine hydrochloride, 0.25 mg; folic acid, 0.2 mg; biotin, 0.02 mg; vitamin B_{12}, 0.01 mg; choline bitartrate, 200.0 mg; inositol, 100.0 mg; and *p*-aminobenzoic acid, 10.0 mg. The levels of thiamine, riboflavin, pyridoxine, pantothenate and choline used were twice those listed by Brown and Sturtevant ('49) as the levels required by the growing rat. The remaining 6 groups received similar diets containing 30% of the levels of the water-soluble vitamins given to the other animals. The sea salt was donated [1] and the sodium chloride and potassium chloride were reagent grade chemicals.

[1] Trace Elements Corporation, Houston, Texas.

During the 17-week experimental period, the animals were individually housed in screen bottom cages in an air-conditioned room maintained at 74° to 76°F. The diets and tap water were provided ad libitum, and the animals were weighed individually at weekly intervals. At the end of the experiment, the animals were fasted for 24 hours, sacrificed by intraperitoneal injection of Nembutal [2] solution, and the livers, kidneys, hearts and adrenals were removed and weighed. The eviscerated carcass of each animal was weighed in a tared Mason jar, autoclaved at 121°C for 4 hours, and homogenized in a Waring blendor. Samples were taken for determination of moisture and total lipid (ether-soluble material) by the method of Sarett and Jandorf ('47) and of nitrogen by the Kjeldahl method. Protein (N × 6.25) and total lipid values were calculated on a fresh weight basis. Moisture content was obtained as the difference between wet weight and dry weight, and is expressed both as a percentage of the fresh weight and as a percentage of the fat-free fresh weight. The kidneys of two representative animals fed each diet were fixed in Zenker's solution and examined histologically, after staining with hematoxylin and eosin. The data of the experiment were subjected to appropriate statistical analysis by the methods outlined by Snedecor ('55).

RESULTS AND DISCUSSION

Data on average weight gains, ad libitum food and water intakes and caloric efficiency values obtained during the experiment are summarized in table 1, and curves of weight gain are given in figure 1. Caloric efficiency values (grams gain per 1000 Cal. consumed) were determined, rather than food efficiency values, because the diets containing added salt were somewhat lower in caloric value than the control diets.

The animals fed diet 7, the control diet which contained adequate levels of B vitamins, gained 320 gm during the experiment, with a caloric efficiency of 44.0, whereas those which

[2] Abbott.

TABLE 1

Data on weight gains, ad libitum food and water intakes and caloric efficiencies of male weanling rats fed otherwise adequate diets containing different levels of B vitamins, sodium chloride, potassium chloride and sea salt for 17 weeks

	DIET NO. AND DESCRIPTION	NO. OF SURVIVORS [1]	INITIAL WEIGHT	GAIN IN WEIGHT	FOOD INTAKE	WATER INTAKE		CALORIC EFFICIENCY [2]
			gm	*gm*	*gm*	*ml*	*ml/100 gm food*	
Inadequate B vitamins								
1	Control	10	45	149 ± 42 [3]	1057	1331	126	32.2 ± 7.1
2	+ 3% NaCl	9	45	118 ± 54	930	1997	215	30.1 ± 10.8
3	+ 6% NaCl	10	45	92 ± 33	941	2861	304	25.2 ± 8.5
4	+ 4% sea salt	9	45	78 ± 31	760	1675	220	25.3 ± 7.9
5	+ 8% sea salt	8	45	111 ± 68	975	3854	395	28.3 ± 9.8
6	+ 6% NaCl + 2% KCl	6	45	92 ± 35	972	3221	331	24.9 ± 9.5
Adequate B vitamins								
7	Control	10	47	320 ± 32	1753	2068	118	44.0 ± 5.0
8	+ 3% NaCl	10	48	307 ± 37	1776	4181	235	42.9 ± 3.5
9	+ 6% NaCl	10	47	305 ± 62	1806	6166	341	42.8 ± 8.3
10	+ 4% sea salt	9	47	319 + 28	1686	3872	230	47.0 ± 2.4
11	+ 8% sea salt	10	47	290 ± 34	1783	7391	415	42.9 ± 5.8
12	+ 6% NaCl + 2% KCl	10	48	294 ± 27	1859	6746	363	41.3 ± 4.3

[1] Each group contained 10 animals initially.

[2] Grams gain per 1000 Cal.

[3] Standard deviation.

received the control diet deficient in B vitamins (diet 1) gained only 149 gm, with a significantly lower caloric efficiency of 32.2 ($P < 0.01$, by "t" test). These adverse effects of vitamin deficiency on weight gain and caloric efficiency were also observed during the earlier weeks of the experiment (see fig. 1).

In the animals which received adequate amounts of B vitamins (diets 7 to 12), sodium chloride, sea salt or the combina-

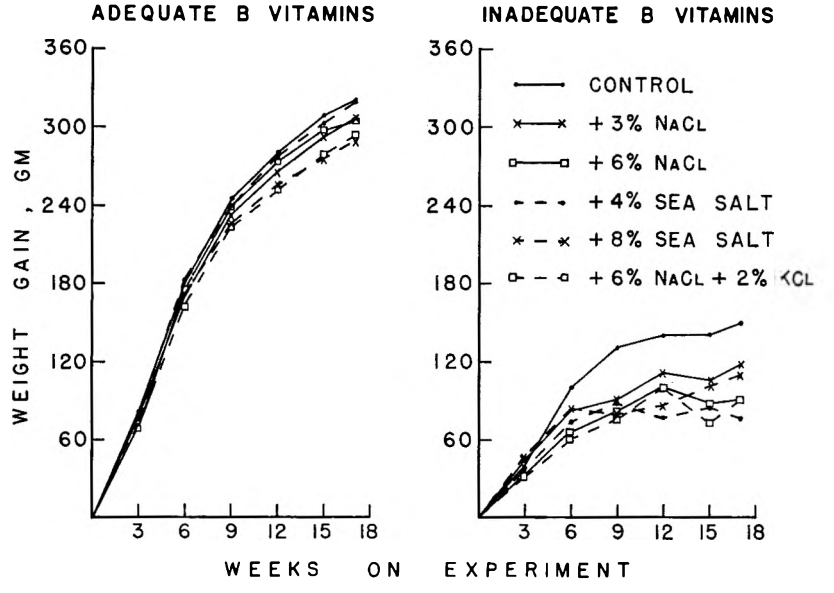

Fig. 1 Effect of excess salt on weight gains of male weanling rats fed diets containing adequate or inadequate levels of B vitamins.

tion of sodium and potassium chlorides had no significant effects on weight gain or caloric efficiency. However, addition to the vitamin-deficient control diet of 3 or 6% of sodium chloride (diets 2 and 3), or equivalent amounts of sea salt (diets 4 and 5), resulted in significantly reduced weight gain ($P < 0.01$) and somewhat lower caloric efficiency. The adverse effects of 6% of sodium chloride on the weight gain and caloric efficiency of the vitamin-deficient animals, were not influenced by addition of 2% of potassium chloride to the diet (diet 6).

Polydipsia and polyuria were observed in the animals which received the diets containing added salt. The animals which received diets containing 3% of sodium chloride (diets 2 and 8) consumed amounts of water per 100 gm of food intake comparable to those which received similar diets containing 4% of sea salt (diets 4 and 10). However, the animals fed the two diets containing 8% of sea salt (diets 5 and 11) consumed more water per 100 gm food intake than those fed the corresponding diets containing 6% of sodium chloride (diets 3 and 9), or 6% of sodium chloride and 2% of potassium chloride (diets 6 and 12).

The data on organ weights are summarized in table 2. The livers, kidneys, and hearts of the animals which received the vitamin-deficient control diet (diet 1) were somewhat larger per unit of body weight, than those of the animals fed the adequate control diet (diet 7). These differences are probably related, in part, to the slower rate of growth and smaller body size of the animals fed the vitamin-deficient diet. The marked adrenal hypertrophy observed in the vitamin-deficient animals is also probably a reflection of the stress of vitamin deficiency.

In the vitamin-deficient animals, but not in those fed adequate amounts of B vitamins, addition to the control diet of sodium chloride, sea salt or the mixture of sodium chloride and potassium chloride, resulted in somewhat larger livers and adrenals, per unit of body weight.

Addition to the vitamin-deficient control diet of 3 or 6% of sodium chloride, equivalent amounts of sea salt, or 6% of sodium chloride and 2% of potassium chloride, resulted in similar significant increases in the relative weight of the kidneys ($P < 0.01$). However, in the animals which received adequate B vitamins, the lower levels of sodium chloride and sea salt had no significant effect on kidney size, although the higher levels of the two substances, and the mixture of 6% of sodium chloride and 2% of potassium chloride, significantly increased the relative size of the kidneys ($P < 0.01$). Histological examination of the kidneys of two animals fed each

TABLE 2

Data on liver, heart, kidney and adrenal weights of male weanling rats fed otherwise adequate diets containing different levels of B vitamins, sodium chloride, potassium chloride and sea salt for 17 weeks

	DIET NO. AND DESCRIPTION	NO. OF SURVIVORS [1]	LIVER		HEART		KIDNEY		ADRENAL	
			gm	*gm/100 gm body weight*	*mg*	*mg/100 gm body weight*	*gm*	*gm/100 gm body weight*	*mg*	*mg/100 gm body weight*
Inadequate B vitamins										
1	Control	10	5.3	2.9 ± 0.2 [2]	612	337 ± 33	1.65	0.90 ± 0.11	35.8	20.1 ± 4.2
2	+ 3% NaCl	9	4.8	3.2 ± 0.5	576	392 ± 59	1.62	1.12 ± 0.23	31.2	22.2 ± 6.1
3	+ 6% NaCl	10	4.2	3.3 ± 0.3	512	409 ± 46	1.53	1.23 ± 0.13	27.6	22.5 ± 4.8
4	+ 4% sea salt	9	3.7	3.3 ± 0.6	479	426 ± 70	1.38	1.24 ± 0.19	27.9	25.5 ± 6.8
5	+ 8% sea salt	8	4.8	3.3 ± 0.3	598	411 ± 47	1.63	1.17 ± 0.17	28.9	21.6 ± 5.7
6	+ 6% NaCl + 2% KCl	6	4.1	3.4 ± 0.6	504	414 ± 81	1.49	1.23 ± 0.21	25.4	21.5 ± 6.5
Adequate B vitamins										
7	Control	10	9.3	2.7 ± 0.2	995	286 ± 27	2.35	0.68 ± 0.09	42.4	12.2 ± 1.7
8	+ 3% NaCl	10	9.1	2.7 ± 0.3	1028	298 ± 34	2.38	0.75 ± 0.07	43.2	13.1 ± 2.0
9	+ 6% NaCl	10	9.3	2.8 ± 0.3	1156	361 ± 93	2.71	0.83 ± 0.09	44.8	14.4 ± 6.5
10	+ 4% sea salt	9	9.2	2.7 ± 0.2	1020	298 ± 31	2.52	0.73 ± 0.07	39.9	11.6 ± 0.8
11	+ 8% sea salt	10	9.3	3.0 ± 0.4	1256	402 ± 77	2.52	0.80 ± 0.08	40.6	13.1 ± 3.5
12	+ 6% NaCl + 2% KCl	10	8.4	2.6 ± 0.2	993	311 ± 31	2.56	0.80 ± 0.06	39.7	12.4 ± 1.2

[1] Each group contained 10 animals initially.

[2] Standard deviation.

diet [3] failed to reveal any marked abnormalities of structure. No evidence of tubular swelling, necrosis or degenerative changes was noted. The kidney tubules were free of epithelial debris, casts and cellular exudates.

In the vitamin-deficient animals, addition to the diet of 3 or 6% of sodium chloride, equivalent amounts of sea salt, or 6% of sodium chloride and 2% of potassium chloride, resulted in similar increases in the relative weight of the heart. These results are in contrast to the findings with the animals fed adequate B vitamins, in which only the higher levels of sodium chloride and sea salt caused significant cardiac hypertrophy. Cardiac hypertrophy has been observed previously, by other investigators, in rats fed high levels of sodium chloride (Tucker et al., '54; Ball and Meneely, '57). In the animals fed adequate B vitamins, the mixture of 6% of sodium chloride and 2% of potassium chloride resulted in less cardiac hypertrophy than was found with 6% of sodium chloride alone. These results, which indicate an ameliorating effect of potassium chloride on sodium chloride toxicity, are in line with the observations of Meneely et al. ('56), that addition of potassium chloride to the diet increased the survival time of rats fed toxic levels of sodium chloride.

When calculated as a percentage of the fresh weight, the level of carcass moisture (table 3) tended to be greater in the animals fed inadequate amounts of B vitamins (diets 1 to 6), than in those which received adequate amounts of B vitamins (diets 7 to 12), but was not significantly influenced by the level of salt in the diet. However, when calculated on the basis of the fat-free fresh weight, the level of carcass moisture was apparently uninfluenced by the dietary B vitamin or salt intakes of the animals, and tended to be a constant value. These latter results are in agreement with those of Pace and Rathbun ('45) who advanced the concept of a fat-free body mass of relatively constant gross chemical composition.

[3] This was done by Dr. C. C. Erickson, University of Tennessee, Knoxville.

TABLE 3

Data on fasting weight losses and carcass composition of male weanling rats fed otherwise adequate diets containing different levels of B vitamins, sodium chloride, potassium chloride and sea salt for 17 weeks

DIET NO. AND DESCRIPTION	NO. OF SURVIVORS [1]	CARCASS COMPOSITION				
		MOISTURE		Ash	Fat	Protein
		Wet weight	Fat-free weight			
		%	%	%	%	%
Inadequate B vitamins						
1 Control	10	65.7 ± 1.8 [2]	71.0 ± 0.7	4.8 ± 0.4	7.5 ± 2.6	23.5 ± 1.0
2 + 3% NaCl	9	66.7 ± 1.2	70.9 ± 0.7	5.1 ± 1.0	5.9 ± 2.3	24.4 ± 0.6
3 + 6% NaCl	10	67.2 ± 1.0	70.9 ± 1.1	5.2 ± 0.7	5.3 ± 1.4	23.9 ± 0.7
4 + 4% sea salt	9	67.4 ± 1.1	70.7 ± 1.6	5.6 ± 0.6	4.8 ± 1.4	24.2 ± 0.6
5 + 8% sea salt	8	66.4 ± 2.1	70.8 ± 1.5	5.3 ± 0.8	6.2 ± 3.3	23.9 ± 0.9
6 + 6% NaCl + 2% KCl	6	66.1 ± 1.1	70.8 ± 0.9	5.3 ± 0.7	6.1 ± 2.0	23.9 ± 1.0
Adequate B vitamins						
7 Control	10	61.7 ± 2.7	71.2 ± 0.7	3.7 ± 0.3	13.4 ± 3.3	22.7 ± 0.9
8 + 3% NaCl	10	61.7 ± 1.3	71.0 ± 0.4	3.8 ± 0.2	13.1 ± 1.7	23.0 ± 0.6
9 + 6% NaCl	10	62.2 ± 3.0	71.0 ± 0.4	4.0 ± 0.6	12.3 ± 4.5	23.1 ± 1.3
10 + 4% sea salt	9	60.0 ± 2.5	71.1 ± 0.4	3.7 ± 0.4	15.6 ± 3.4	22.2 ± 1.0
11 + 8% sea salt	10	63.5 ± 2.0	71.1 ± 1.0	3.8 ± 0.2	11.0 ± 2.3	23.0 ± 0.7
12 + 6% NaCl + 2% KCl	10	61.0 ± 1.8	70.9 ± 0.5	3.7 ± 0.2	14.0 ± 2.1	22.7 ± 0.5

[1] Each group contained 10 animals initially.

[2] Standard deviation.

The level of carcass fat was approximately twice as high in the animals which received adequate amounts of B vitamins as in those which received the vitamin-deficient diets and was not significantly influenced by the level of salt in the diet.

Carcass ash values were significantly higher in the animals fed the vitamin deficient control diet (diet 1) than in those fed the control diet containing adequate B vitamins ($P < 0.01$). This difference is probably related to the greater skeleton to body weight ratio found in the smaller vitamin-deficient animals. No effects of the salt level of the diet on carcass ash values were seen in the animals which received sufficient B vitamins, whereas in those receiving vitamin-deficient diets, the carcass ash values were slightly, but not significantly, increased by the addition of sodium chloride, sea salt or the mixture of sodium and potassium chlorides to the diet.

The results of the present experiment indicate that the toxicity of excess sodium chloride or sea salt is markedly influenced by the B vitamin intake. The small vitamin-deficient animal is evidently less able to withstand the additional stress of high dietary salt levels than is the animal which has received adequate B vitamins. Other investigators have also shown that nutritional factors are involved in the ability of animals to withstand stress (Dumm and Ralli, '50; Ershoff, '52).

SUMMARY

An experiment was conducted to determine the effects of B vitamin deficiency on the toxicity of excess salt for the growing rat.

During a 17-week experimental period, the weight gain and caloric efficiency of animals which received an otherwise adequate diet deficient in B vitamins were significantly less than those of animals fed a similar diet containing adequate amounts of B vitamins. In the vitamin-deficient animals, but not in those which received adequate B vitamins, addition to the diet of 3 or 6% of sodium chloride, or equivalent amounts of sea salt (4 and 8%) resulted in significantly decreased weight gain and somewhat lower caloric efficiency.

Addition to the vitamin-deficient diet of 3 or 6% of sodium chloride, or equivalent amounts of sea salt, resulted in similar significant increases in the relative weight of the kidneys, adrenals and hearts of the animals. However, in the animals which received adequate B vitamins, only the higher levels of sodium chloride and sea salt caused significant renal and cardiac hypertrophy. The cardiac hypertrophy brought about in the normal animals by addition of 6% of sodium chloride to the diet was prevented to a large extent by concomitant administration of 2% of potassium chloride.

The level of carcass moisture (calculated on a fat-free basis) tended to be a constant value and was uninfluenced by the dietary B vitamin or salt intake.

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THE SPECIFICITY OF THE MOLYBDATE-SULFATE INTERRELATIONSHIP IN RATS [1]

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Since the original observation of Ferguson, Lewis, and Watson ('38) who associated a scouring disease of cattle in certain areas of England with a high molybdenum content of the pastures, several studies on molybdenum toxicosis in laboratory animals have been reported (Neilands et al., '48; Comar et al., '49; Arrington et al., '53; Gray et al., '54). It was subsequently demonstrated that dietary sodium sulfate can ameliorate the effects of toxic levels of sodium molybdate in the rat (Van Reen and Williams, '56). Although the interrelationship has been substantiated by several groups (Miller et al., '56; Mills et al., '58), the mechanism of the action is not clear and the specificity of the sulfate effect has not been reported. The present study indicates that sulfate has a fairly specific action in that other anions such as citrate, tartrate, acetate, bromide, chloride, and nitrate do not mitigate the toxicity of molybdate. The effects of several molybdenum salts are also reported.

METHODS

Albino rats of the Wistar strain were used for these studies, except where indicated. The basal diet had the following per-

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

centage composition: casein, 20.0; glucose, 69.0; corn oil, 6.6; U.S.P. XIV salt mixture, 4.0; choline chloride, 0.1; 2500 I. U. of vitamin D, and 15 mg α-tocopherol in corn oil, 0.3. Other vitamins were provided in adequate quantities as reported previously (Van Reen and Williams, '56). In the studies on the influence of various anions on toxicity and the effects of various molybdenum salts, the molybdenum concentration used was 0.8 mM per 100 gm of diet and that of the various supplements, 2.0 mM per 100 gm of diet. The experimental diets were fed 4 weeks and then the animals sacrificed and the livers assayed for alkaline phosphatase by the method of Bessey, Lowry, and Brock ('46) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol is measured. Each tissue was assayed at two concentrations and each concentration run in duplicate. Activities are expressed as micromoles of phosphate released in 30 min. per milligram protein. The protein content of the homogenates was determined by the method of Lowry et al. ('51). The release of hydrogen sulfide by the action of acid on potassium thiomolybdate was measured by the formation of methylene blue by the procedure of Fogo and Popowsky ('49). The enzyme data and the weight values were analyzed statistically and values are reported with standard errors of the means.

RESULTS AND DISCUSSION

The influence of various anions on molybdate toxicity in rats is shown in table 1. Previous observations (Van Reen, '54) on the growth-depressing action of 0.8 mM sodium molybdate per 100 gm of diet were substantiated as was the effect of this level of molybdate in increasing the activity of liver alkaline phosphatase. The supplementation of the toxic diet with 2.0 mM of sodium sulfate resulted in a significant improvement in growth ($P < 0.01$) and a significant reduction in the activity of liver alkaline phosphatase ($P < 0.01$). On the other hand, supplementation of the toxic diet with sodium citrate, tartrate, acetate, bromide, chloride, or nitrate neither

improved the growth nor brought about a reduction in liver alkaline phosphatase. Rather, the anions other than sulfate resulted in higher alkaline phosphatase activities. Thus, not all anions have an ameliorating effect on molybdenum toxicosis and the sulfate interrelationship may be fairly specific.

TABLE 1

Influence of various anions on molybdate toxicity in rats [1]

SUPPLEMENT	BODY WEIGHT	LIVER ALKALINE PHOSPHATASE
	gm	*μM phosphate released/ 30 min./mg protein*
None	195 ± 8.8 [2]	0.083 ± 0.004 [2]
Na_2MoO_4	90 ± 5.7	0.161 ± 0.020
Mo + Na_2SO_4	153 ± 4.6	0.113 ± 0.010
Mo + Na_3 citrate	102 ± 4.7	0.200 ± 0.027
Mo + Na_2 tartrate	88 ± 7.8	0.258 ± 0.028
Mo + Na acetate	104 ± 6.3	0.224 ± 0.041
Mo + NaBr	98 ± 5.4	0.223 ± 0.022
Mo + NaCl	95 ± 3.7	0.227 ± 0.023
Mo + $NaNO_3$	82 ± 4.4	0.225 ± 0.071

[1] Ten animals per group.
[2] Standard error of the mean.

The effects of various molybdenum salts with and without sodium sulfate are presented in table 2. It was observed again that sodium molybdate caused a significant reduction in the growth responses and that similar results were obtained with equal molar concentrations of molybdenum trioxide and molybdenum pentachloride. Similarly, these three compounds caused increased liver alkaline phosphatase. Sodium sulfate added to the toxic diets was able to mitigate the growth depression and the elevated liver alkaline phosphatase activity. The correction of the toxicosis by sulfate was highly significant in all cases ($P < 0.01$). These studies, however, do not exclude the possibility of a specific molybdate-sulfate anion interrelationship since the conversion of molybdenum trioxide and molybdenum pentachloride to molybdate are likely reactions.

Potassium thiomolybdate was extremely toxic and all rats died by the end of 4 weeks. The effects of the thiomolybdate were not influenced by supplementation with sodium sulfate. Because of the extreme toxicity of the thiomolybdate, a study of the release of hydrogen sulfide by 0.1 N HCl *in vitro* was made. It was found that one-half the theoretical sulfur content of potassium thiomolybdate was released under acid conditions. Thus, part of the toxicity was probably due to sulfide. In this regard, it appears that molybdenum salts are more

TABLE 2

Effect of various molybdenum salts and sulfate[1]

SUPPLEMENT	BODY WEIGHT	LIVER ALKALINE PHOSPHATASE
	gm	*μM phosphate released/ 30 min./ mg protein*
None	238 ± 17[2]	0.067 ± 0.005[2]
MoO_3	130 ± 4	0.166 ± 0.026
$MoO_3 + Na_2SO_4$	210 ± 9	0.097 ± 0.009
$MoCl_5$	156 ± 11	0.136 ± 0.019
$MoCl_5 + Na_2SO_4$	204 ± 5	0.070 ± 0.003
Na_2MoO_4	128 ± 11	0.176 ± 0.021
$Na_2MoO_4 + Na_2SO_4$	199 ± 8	0.084 ± 0.006
K_2MoS_4	all dead	
$K_2MoS_4 + Na_2SO_4$	all dead	

[1] Ten animals per group.
[2] Standard error of the mean.

toxic in the presence of sulfide. Mills ('58) recently reported that sulfide was not effective in preventing the development of molybdenum toxicosis in the rat although sulfide normally can be oxidized to sulfate. Low levels of sulfide, on the contrary, were quite toxic if tissue levels of molybdenum were elevated (Mills, '58). This may be a reflection of the depressed liver sulfide oxidase activity found in rats administered sodium molybdate (Mills et al., '58).

Since the molybdate-sulfate relationship appears specific, further studies were performed to determine whether lower levels of molybdate would be toxic if all sulfate were removed from the salt mixture. To accomplish this a modified U.S.P.

XIV salt mixture was formulated in which chlorides or carbonates replaced the sulfate salts. The results of this experiment are presented in table 3 and demonstrate that while good growth was obtained on the basal diet containing the sulfate-free salt mixture, the inclusion of 50 μM of sodium molybdate/100 mg of diet resulted in smaller animals. Thus, it appears that as little as 5 mg molybdenum/100 gm of diet can have pronounced effects when the sulfate concentration is low.

TABLE 3

Effect of a low level of sodium molybdate on NMRI-D strain[1] of albino rat fed a sulfate-free diet[2]

SUPPLEMENT	BODY WEIGHTS	
	Start	5 weeks
	gm	*gm*
None	30 ± 1[3]	181 ± 4[3]
Na_2MoO_4 (50 μMoles/100 gm)	30 ± 1	155 ± 8

[1] Losee and Gerende ('57).
[2] Twenty animals per group.
[3] Standard error of the mean.

During the course of the above studies, observations were made on the skeletal development. It was noticed that many rats of the NMRI-D strain (Losee and Gerende, '57) developed mandibular or maxillary exostoses when fed the molybdenum toxic diets, whereas none of the control rats developed the syndrome. Figure 1 shows the appearance of the exostoses. The maxillary exostoses were not as severe as the mandibular and were limited to the superior surface of the anterior portion of the zygomatic arch. The most pronounced exostoses occurred on the anterior aspect of the masseteric ridge of the mandible. Smaller growths were frequently seen on the posterior portion of the ridge and also on the distal portion of the condyloid and coronoid processes. In almost all cases the exostoses were limited to the lateral surfaces of the mandible and maxilla.

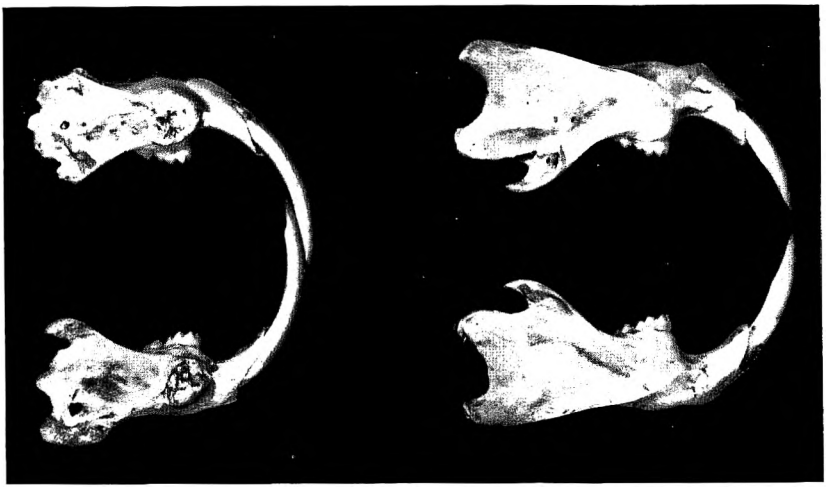

Fig. 1 Photograph of lateral aspect of mandibles from control animals (pair on right) and from molybdenum toxic animals (pair on left). Note the severe exostoses on the anterior portion of the masseteric ridge and the roughness of the posterior portion of the ridge and processes.

The occurrence of exostoses in one experiment is shown in table 4. It can be seen that one-half of the rats receiving 0.4 mM molybdate/100 gm of diet developed mandibular exostoses whereas rats receiving 2.0 mM sodium sulfate in addition to the molybdate showed a reduced tendency toward the condition. Only one animal on the low level of molybdate developed abnormal bone growths. These observations demonstrate another biological response of the rat to dietary molybdenum and also indicate that inorganic sulfate can have an ameliorating effect on the toxicity. Further studies will be performed to determine whether the formation of exostoses can be completely eliminated by increasing the level of sulfate.

TABLE 4

Occurrence of mandibular exostoses in the NMRI-D rat [1]

	SUPPLEMENT IN mM/100 GM OF BASAL DIET			
	None	0.4 Mo	0.4 Mo + 2.0 SO_4^{--}	0.05 Mo
No. rats with exostoses	0	10	5	1

[1] Twenty animals per group.

SUMMARY

1. The specificity of the molybdate-sulfate interrelationship in rats was investigated. It was found that sodium sulfate was able to alleviate the effects of molybdenum toxicity, whereas sodium citrate, tartrate, acetate, bromide, chloride, and nitrate did not have this property.

2. A condition similar to sodium molybdate toxicity was obtained by feeding molybdenum trioxide or molybdenum pentachloride and in all cases the toxicity was mitigated by sodium sulfate.

3. Potassium thiomolybdate produced an extreme toxicosis, presumably through the release of hydrogen sulfide which is believed to potentiate molybdenum toxicity. This condition was not ameliorated by sodium sulfate.

4. As little as 5 mg of molybdenum per 100 gm of diet resulted in reduced growth of NMRI-D weanling rats when the sulfate level of the diet was low.

5. Mandibular and maxillary exostoses were observed in rats receiving dietary molybdate. The characteristics of the bony proliferations are described.

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THE ANTITHYROTOXIC FACTOR OF LIVER

II. COMPARATIVE ACTIVITIES OF DEFATTED LIVER RESIDUE AND VARIOUS FATS

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Recently, Overby et al. ('58) described methods used to test responses of thyrotoxic rats to liver residue. The protective action of liver residue was found independent of vitamins, minerals, casein, fats, carbohydrates, antibiotics and inert ingredients. Penicillin and fats exerted a growth increment in both normal and thyrotoxic rats. However, they did not alter the usual response to liver residue.

Most of the previous studies were carried out with the petroleum ether-extracted fraction of the water-insoluble portion of hog liver. The whole liver residue is about 75% protein and about 20% lipid. Defatted residue, as well as the lipid fraction, has antithyrotoxic activity (Page et al., '56, Stevens and Henderson, '58).

Ershoff ('49, '53) has reviewed the earlier studies which showed that an increased intake of dietary fat exerts a beneficial effect on the hyperthyroid animal. He, also, has found a direct correlation between the fat content of the diet and growth of hyperthyroid rats. Under his experimental conditions cottonseed oil at 10% of the diet promoted growth and survival over that found on a fat-free diet. Defatted liver residue was active in a fat-free diet and also in the presence of cottonseed oil, but large amounts of B vitamins were active

only in the presence of cottonseed oil. The fats, however, counteracted growth depression only, and not the other effects of thyroid administration.

Greenberg ('52) and Greenberg and Deuel ('50) showed that the growth-depressing effects of excess thyroxine may be partially counteracted by cottonseed oil or methyl linoleate. Emerson et al. ('56) and Page et al. ('56) have also shown that unsaturated fats and certain steroids were active in a thyroid stress assay. However, the entire activity of liver did not appear to be accounted for by these compounds alone.

The requirements for almost all nutrients are altered in the thyrotoxic animal. With the exception of crude liver residue, fats seem to be the most specific antithyrotoxic food substances. The present studies compare the protective effects of defatted liver residue, liver fat, and 6 processed fats of widely different composition.

EXPERIMENTAL AND RESULTS

The experimental techniques and diet composition are described fully in the previous publication (Overby et al., '58). The gross composition of diet 14 used in these experiments was: 30% protein, 5% fat, 57.5% carbohydrate, 4% salts, 3.25% inert ingredients and 0.25% vitamins. Thus 30.4% of the calories were furnished by protein, 11.4% by fat, and 58.2% by carbohydrate. The experimental diets were made by substituting the test material for the appropriate nutrient, providing diets as isonitrogenous and isocaloric as possible. If fats were tested at a level higher than 5%, all over this amount was added for an equal weight of carbohydrate. This increased the over-all caloric value of the diet and the percentage of calories supplied from fat.

Iodinated casein [1] was added on a caloric basis. The basal 5% fat ration with 0.35% of iodinated casein provided 3.95 Cal. per gram. The ratio of calories to milligrams of iodinated casein was thus 3.95 to 3.5, a ratio which was maintained when the caloric equivalent of the diet varied.

[1] Protamone, Cerophyl Laboratories, Kansas City, Mo.

Experimental Series 1

Comparison of whole and defatted commercial liver residue

Commercial liver residue (Overby et al., '58) represents the water-insoluble fraction of liver reported by various laboratories to have high antithyrotoxic activity. It contains about 20% of ether extractable material. In a series of 24 experiments we investigated the non-defatted liver residue and various solvent extracts of it. A summation of the results is shown in table 1. The solvents used were petroleum ether, chloroform, ethylene dichloride, ethanol and an azeotropic

TABLE 1

Antithyrotoxic activity of commercial liver residue fat and defatted fractions

GROUP	IODINATED CASEIN [1]	SUPPLEMENT TO DIET 14	5-WEEK RESULTS [2]	
			Gains	Surviving
	%		*gm* *S.E.*	% *S.E.*
1	0	None	194 ± 4.7	100
2	0.35	None	125 ± 3 9	50 ± 6.0
3	0.35	10% Whole liver residue	168 ± 4.6	90 ± 1.8
4	0.35	2.5% Liver residue fat	147 ± 5.2	90 ± 2.8
5	0.35	7.5% Defatted liver residue	163 ± 3.7	90 ± 1.9

[1] Protamone.

[2] The mean gains and survivals from 24 experiments. Ten rats were used in each group in each experiment. S.E. is standard error of the mean between experiments $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

mixture of chloroform-methanol. The extracts were prepared by exhaustive extraction in a Soxhlet type apparatus. The amount extracted varied from 18% with petroleum ether to 25% with chloroform-methanol. The solvents were removed from the extracts by distilling to a low volume and then heating at 60°C, 15 mm pressure, while bubbling in nitrogen. The extracted residue was freed of solvent by drying in air and then heating at 60°C in a vacuum oven at 15 mm pressure. The fat and the defatted residue were assayed at 2.5% and 7.5%, respectively. The fat was substituted in diet 14 for an

equal weight of cottonseed oil and the defatted residue for an equal weight of casein. Each of the fat extracts was active, but less so than the liver residue. There were no differences in the activity of fractions extracted by the various solvents, and in each case no significant loss of activity could be detected in the defatted liver residue. In the 24 tests the average gain was 168 gm for the whole liver residue, 147 gm for the fat fractions and 163 gm for the defatted residue.

These results indicated also that liver lipids were more active than an equal weight of cottonseed oil. In table 1 the diets for groups 1, 2 and 5 contained 5% of cottonseed oil and groups 3 and 4 diets contained 2.5% of cottonseed oil plus 2.5% of liver lipids. It was also evident that most of the antithyrotoxic activity was associated with the predominantly protein fraction of liver residue.

Experimental series 2
Antithyrotoxic activity of autolyzed liver residue fat and defatted fractions

One possible interpretation of the foregoing experimental series is that a factor in liver is bound to protein and when released is soluble in the liver fat and fat solvents. Further, in commercial liver residue release of bound material is only partial. To test this idea and to determine if more activity could be released to the lipid fraction, fresh pork liver was autolyzed and the soluble and insoluble fractions recovered.

For the autolysis 35 lbs. of fresh frozen hog liver were thawed and ground in an electric meat grinder. This was added to 64 l of deionized water, containing 130 gm of cysteine hydrochloride and 2 l of toluene. Nitrogen was bubbled through the mixture as it was homogenized by vigorous mechanical stirring. The pH was adjusted to 3.9 with concentrated HCl (about 200 cm^3). The mixture was placed in a constant temperature room (40°C) and nitrogen bubbled through at a rate to insure moderate stirring of the contents. The autolysis was continued for 6 days, during which the pH was readjusted to

4.0 daily with concentrated HCl. The mixture was then heated at boiling for one hour by passing steam through a submerged copper coil. After standing overnight, the supernatant was decanted through canvas on a Buchner funnel. The insoluble portion was transferred to the funnel, washed with water and dried at 100°C. The yield of dried residue was 1200 to 1400 gm. Portions of the filtrate were freeze-dried for testing.

Table 2 summarizes the averaged results from 10 experiments in which defatted commercial liver residue, lyophilized fresh hog liver, the autolysis liver residue and the liver auto-

TABLE 2

Comparison of antithyrotoxic activity of defatted liver residue and liver residue after autolysis

GROUP NO.	IODINATED CASEIN [1]	SUPPLEMENT TO DIET 10–13	4-WEEK RESULTS [2]			
			Gains		Survival	
	%		*gm*	*S.E.*	%	*S.E.*
1	None	None	147	± 5.5	100	
2	0.35	None	78	± 7.4	40	± 4.6
3	0.35	10% Defatted liver residue	123	± 8.6	90	± 4.5
4	0.35	10% Lyophilized whole liver	98	± 5.9	70	± 9.0
5	0.35	5% Autolysis liver residue	135	± 11.6	90	± 5.0
6	0.35	10% Lyophilized liver autolysate	79	± 14.2	50	± 8.7

[1] Protamone.

[2] The mean gains and survivals from 10 experiments. Ten rats were used in each group in each experiment. S.E., see footnote 2, table 1.

lysate solids were compared in the rat antithyrotoxic assay. The lyophilized fresh liver was active but was less potent than an equal quantity of defatted commercial liver residue. This was to be expected because the active portion had not been concentrated by removal of the water-soluble substances. There was no activity in the portion of liver solubilized by autolysis. The insoluble portion from the autolysis was at least twice as potent as commercial liver residue (group 5 vs. group 3). The animals in groups 1, 2, 3 and 6 consumed diets containing 5% of cottonseed oil, while the diets of groups 4 and 5 contained 2.5% of cottonseed oil plus the fats present in the supplement (about 2.5%).

TABLE 3

Antithyrotoxic activity of fat and non-fat fractions of autolysis liver residue

GROUP	NO. OF EXPS.	IODINATED CASEIN [1]	SUPPLEMENT TO DIET 10–13	4-WEEK RESULTS [2]	
				Gain	Survival
		%		*gm* *S.E.*	% *S.E.*
1	30	0	None	152 ± 3.6	100
2	32	0.30	None	76 ± 4.8	30 ± 4.7
3	30	0.30	10% Defatted liver residue	121 ± 5.1	80 ± 9.1
4	22	0.30	5% Autolysis liver residue	128 ± 4.0	90 ± 3.2
5	30	0.30	2.5% Defatted autolysis liver residue	110 ± 3.3	70 ± 3.7
6	23	0.30	2.5% Autolysis liver residue fat fractions	114 ± 5.8	70 ± 6.4
7	11	0.30	2.5% Defatted autolysis liver residue + 2.5% autolysis liver residue fat	129 ± 2.9	90 ± 2.7
8	2	0.30	5% autolysis liver residue fat	138	70
9	2	0.30	5% Defatted autolysis liver residue	133	90

[1] Protamone.

[2] The mean gains and survivals from the number of experiments recorded in column 2. Ten rats were used in each group in each experiment. S.E., see footnote 2, table 1.

In a series of 32 experiments, the autolysis residue was continuously extracted with petroleum ether, ethanol, ethylene dichloride, or an azeotropic chloroform-methanol mixture. These solvents removed 40 to 55% of the autolysis residue. The antithyrotoxic activity found with the various fractions is shown in table 3. There were no differences in activity of the extracts prepared with the different solvents. All data for the extracts are combined to give average values for the fat fraction and defatted residue.

The fat fraction and the defatted autolysis residue were equally active at the 2.5% level, but each was less active than the whole material. Recombination of the two in equal amounts or increasing either to 5% gave responses equal to that of the whole autolysis residue.

Like the commercial product, both the fat and the non-fat fractions of the autolysis residue were active. The effects of each appeared to be additive and both appeared to be approximately twice as potent, on a weight basis, as the commercial defatted liver residue. Certainly, autolysis did not appear to "release" *all* the activity to the fat fraction. The possibility appeared here also that we were dealing with at least two active materials, one fat soluble the other insoluble in either water or fat solvents. This early indication of multiple factors of widely different chemical nature was clarified, at least in part, by the following experiments with various fats.

Experimental series 3
Antithyrotoxic activity of various refined fats and oils

In a series of 13 experiments cottonseed oil, safflowerseed oil, hydrogenated coconut oil,[2] olive oil, peanut oil, butterfat, and liver fat were tested alone and combined at levels up to 22.5% of the diet. The 5-week weight gain and survival responses are shown in table 4. It was apparent that all fats tested, with the exception of hydrogenated coconut oil promoted growth of

[2] Hydrol, Proctor and Gamble Co., Cincinnati, Ohio.

TABLE 4

Comparative activity of fats with and without liver residue

GROUP	NO. OF EXPS.[1]	FATS [2] ADDED TO DIET 14	LINOLEIC ACID	5-WEEK RESULTS [3]			
				Gain		Survival	
			gm/100 gm diet	*gm*	*S.E.*	%	*S.E.*
Without liver residue and without iodinated casein [4]							
1	2	None	0	201	± 5.9	100	
2	12	5% CSO	2.5	221	± 3.8	100	
3	1	20% SSO	14.0	220		100	
Without defatted liver residue and with iodinated casein (3.5 mg/3.95 Cal.)							
4	3	None	0	90	± 6.5	20	± 11.4
5	1	5% HCO	< 0.2	91		70	
6	2	15% HCO	< 0.6	95		70	
7	3	15% Butterfat	0.6	124	± 8.7	20	± 14.0
8	12	5% CSO	2.5	135	± 5.0	30	± 5.5
9	1	5% CSO + 17.5% HCO	3.1	140		70	
10	1	15% PO	3.2	156		70	
11	3	15% OO	1.1	158	± 13.9	50	± 17.3
12	1	13.8% CSO	6.9	170		60	
13	2	15% CSO	7.5	172		70	
14	3	5% CSO + 8.8% SSO	8.6	172	± 7.3	70	± 11.5
15	3	15% SSO	10.1	176	± 8.4	80	± 13.0
16	3	5% CSO + 17.5% SSO	14.8	175	± 3.0	90	± 6.0
17	1	5% LF	0.5	172		70	
18	1	15% LF	1.5	192		100	
With defatted liver residue (10% of diet) and iodinated casein (3.5 mg/3.95 Cal.)							
19	2	None	0	161		100	
20	1	3% HCO + 2% LF	0.2	162		100	
21	7	3% CSO + 2% LF	1.7	190	± 4.8	90	± 3.1
22	1	15% OO	1.1	194		100	
23	6	5% CSO	2.5	201	± 7.8	90	± 5.0
24	1	15% CSO	7.5	196		100	
25	1	20% SSO	14.0	204		100	

[1] Ten rats per experiment.

[2] CSO = cottonseed oil; SSO = safflower seed oil; HCO = hydrogenated coconut oil; PO = peanut oil; OO = olive oil; LF = liver fat.

[3] The mean gains and survival from the number of experiments recorded in column 2. S.E., see footnote 2, table 1.

[4] Protamone.

thyrotoxic rats. Furthermore, growth was enhanced even more when liver residue preparations were included along with the various levels of fats.

The actual growth-promoting effects of the various fats with and without liver residue may be presented in clearer perspective when weight gain is compared with the amount of linoleic acid supplied by the various fats in the experimental diets. Figure 1 shows the results of table 4 in graphic form,

and clearly demonstrates the growth-promoting effects of linoleic acid for thyrotoxic rats. It is of interest to note that no growth increment occurred beyond about 8% linoleic acid in the diet (curve 1). As shown in curve 2, growth reached a maximum at about 2 to 3% of linoleic acid in the presence of non-fat liver residue. Growth did not reach that of the control

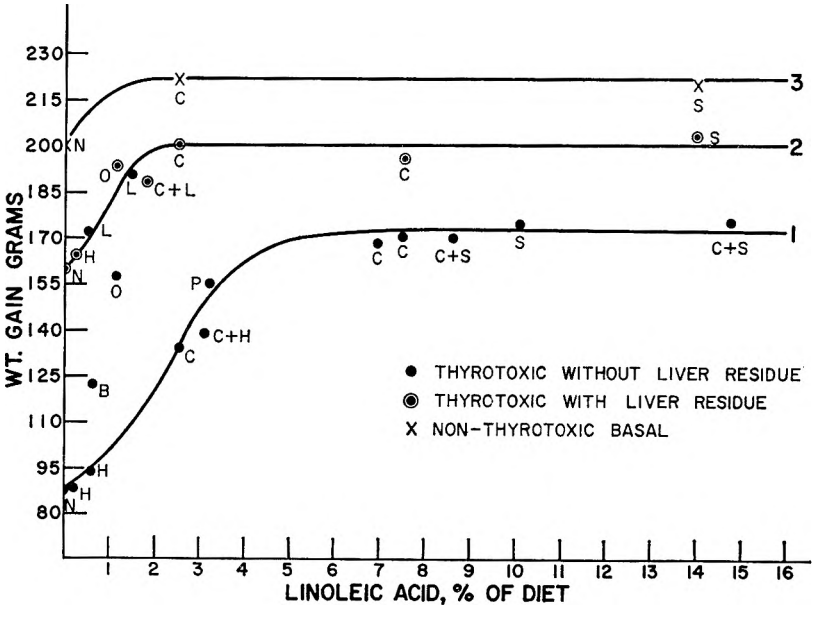

Fig. 1 The effects of linoleic acid supplied by fats on growth of hyperthyroid rats with and without defatted liver residue. N is fat-free diet, H is hydrogenated coconut oil, B is butterfat, O is olive oil, C is cottonseed oil, P is peanut oil, S is safflower seed oil and L is the petroleum ether extract of liver residue.

(curve 3) even on the highest fat diets. It was especially obvious that liver fat fractions fitted the liver residue curve better than the "fat alone" curve. Olive oil and butterfat were conspicuous because growth was better than expected from their linoleate content.

The average survival in the butterfat groups was only 20%, and the larger animals happened to be the survivors at 5 weeks. At the 4-week interval when more animals were living

the weight gain on 15% butterfat was always much lower than that of animals receiving 5% cottonseed oil, as would be expected from the low linoleate content of butterfat. Therefore, based on survival and 4-week weight gains there were no special antithyrotoxic properties of butterfat. However, throughout all experiments, olive oil promoted growth and survival better than expected from the linoleate content alone.

DISCUSSION

This study clearly shows the protective action of certain fats in promoting growth and survival of thyrotoxic rats. The idea of an induced requirement for unidentified nutrients by thyrotoxicosis must always be considered in relation to the antithyrotoxic properties of fats. Fats supply two requirements for the growing animal, a concentrated source of calories and essential nutrients required for metabolic processes. The hyperthyroid animal wastes energy in the form of heat by oxidations not coupled with phosphorylation, i.e., conservation of energy for muscle use. Thus, from a calorie standpoint alone high-fat diets should favorably effect thyrotoxic animals. In this series of studies we have attempted to minimize such response by feeding the thyroid active material based on the calories supplied per unit of diet. The fact that growth reaches a plateau at about 15% of cottonseed oil in the ration indicates that this type of experiment does minimize the effect of calories.

Food consumption data in experimental series 3 (table 4) show that with ad libitum feeding the rats rather uniformly regulate their caloric intake. Five-week food intakes for groups 2, 4, 8, 23, 16 and 25 averaged 14.4, 18.7, 18, 16.9, 13.6 and 13.2 gm per rat per day respectively. These same groups thus ingested iodinated casein at average rates of 0, 62, 63, 59, 59 and 57 mg per rat per day. The differences in actual iodinated casein consumption were small but roughly inversely proportional to the weight gain. Previous experiments (Overby et al., '59) showed that such small differences in iodinated

casein levels would not give such large differences in growth.[3] In fact, it seems remarkable that the iodinated casein consumption was so uniform in diets of widely different composition. Thus in experiments involving growth studies with orally administered thyroid hormone, the level administered should be keyed to the calorie balance of the diet.

The enormous increase in linoleic acid requirement by thyrotoxic rats is amply demonstrated in these experiments. As a "rule of thumb" about 80 mg of linoleic acid per day per kilogram of body weight is required for the normal growing animal on an otherwise balanced diet. In terms of cottonseed oil this would require about 0.15% of the diet for the animals under experimental conditions used in these studies but, as is seen in figure 1, curve 1, 100 times this amount is required for a plateau in the growth of thyrotoxic rats. The effect of olive oil over the apparent linoleic acid content could be due to the high oleic acid. Zain ('36) found that linoleic acid, but not stearic acid, would prevent reduction of liver glycogen in hyperthyroid female rats. Oleic acid also prevented this, but to a lesser extent.

The results illustrated in curve 2, figure 1 indicate that the major activity in liver is neither linoleic acid, nor a nonspecific fat effect. The residual fat remaining in the solvent-extracted residue was recovered after saponification and amounted to about 1.5%. Spectrophotometric analysis after alkali isomerization by the method of Holman and Hayes ('58) showed the following composition of unsaturated fatty acids: 9.4% dienoic, 1.9% trienoic, 5.2% tetraenoic, 1.0% pentaenoic and 0.7% hexaenoic. Thus when the "defatted residue" was tested at the 10% level it added only about 15 to 20 mg of linoleic acid to 100 gm of diet. In the absence of other fats, the defatted residue gave a weight gain of 160 gm. Cottonseed oil also gave a gain of about 160 gm at the 10% level. However, growth above 175 gm was not obtained even with 22.5% vegetable fats which provided almost 15% of the diet as lino-

[3] As will be reported in subsequent publications there were no differences in the basal metabolic rate of animals in the thyrotoxic groups regardless of weight gains.

leic acid. In comparison, the liver fraction plus 5% of cottonseed oil (providing only 2.5% of linoleic acid) consistently supported a weight gain of 205 gm. The vegetable oils would of course supply only monoenoic, dienoic and trienoic acids. The antithyrotoxic activity of acids more highly unsaturated than linoleic has not been studied.

It was of interest to note that the liver fat when viewed in terms of its linoleate content supported growth predicted by the "liver curve" (2), rather than the "fat alone" curve (1). It would seem that this extra protective action would be due to a low level of the same active components found in the defatted residue. This is borne out by experimental series 1 and 2 in which liver fat from autolyzed liver residue appeared to have a relatively higher activity than that from the commercial product. The major portion of the activity appears, however, to be associated with the non-lipid fraction of liver.

SUMMARY

Unsaturated vegetable fats promoted growth of thyrotoxic rats, an effect which appeared to be directly related to the linoleic acid supplied by the fats. Hydrogenated coconut oil was ineffective and butterfat was marginal. The fat fraction of liver residue was, however, more active than other fats supplying more linoleic acid. The defatted fraction of liver residue promoted growth over and above all levels of fats. Growth stimulation by defatted liver residue and linoleic acid containing fats, or liver fat, appeared to be additive.

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BIOASSAY OF THE NUTRITIONAL QUALITY OF THE PROTEIN OF HUMAN AND COW'S MILK BY RAT GROWTH PROCEDURES

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The problem of the protein requirement of infants artificially fed with cow's milk would be clarified in part if the nutritional value of cow's milk protein in comparison with human milk protein were exactly known. Metabolic studies with infants such as those of Gordon et al. ('37), Barness et al. ('57) and Fomon and May ('57) in which nitrogen retention was compared in infants fed human and cow's milk formulas, have shown little difference in the nutritional value of the proteins. Edelstein and Langstein ('19), in a determination of the biological value of the protein, found human milk superior to cow's milk. Metabolic studies with infants, while supplying the most applicable data, are difficult to perform and are not subject to the rigorous control of an animal assay. The determination of the nutritive value of proteins by animal bioassay has had a wide application to human nutrition.

Animal assays of the protein of human milk have been hampered by the intolerance of the assay animal, particularly the rat, to the high concentration of lactose in human milk. Henry, Kon and Mawson ('50) in a determination of the biological value and the true digestion coefficient of the two milk proteins by the method of Mitchell ('23, '24) experienced difficulty in collecting the diarrheal excreta of the rats fed human milk. Human milk protein yielded a biological value of 88.0 and 81.1, average 85.7 with a digestion coefficient of 79.2.

Values of 93.8 and 75.5, average 84.7, were obtained with cow's milk but the second value was probably low because of the previous feeding of the group with human milk. The digestion coefficient of cow's milk protein was 92.5.

Studies of protein isolated from milk, and thus free of lactose, do not assess the contribution of the non-protein fractions. The possibility that the complex polysaccharides in human milk, which stimulate growth of *L. bifidus*, may effect protein utilization has been suggested by the results of rat growth experiments (György et al., '53). In the present study these difficulties were circumvented by feeding human milk in which approximately half the lactose had been removed by crystallization. In addition, the young rats were partially adapted to the lactose diet by a preliminary feeding period. In a second experiment lactose and other diffusable constituents were removed from both milks by dialysis and comparison made of the isolated proteins.

EXPERIMENTAL

Preparation of protein samples

Experiment 1. Human milk of reduced lactose content was prepared from milk [1] collected by the facility for the collection of human milk established in the Department of Pediatrics of the State University of Iowa (from students' wives, average age, 25 yrs.). Daily collections of the milk were pooled, pasteurized and frozen. As most of the donors had been lactating for a period of two to three months before contributing, the sample was considered to consist of "mature" human milk. A 30-liter pasteurized and frozen composite sample of mature human milk containing 53.8 gm of nitrogen (179 mg/100 ml) was processed to remove lactose and fat. The milk was centrifuged for 30 minutes at room temperature, cooled until the cream layer solidified, and the skim layer separated. The skim milk except for 5 liters was lyophilized, the dried material was suspended in the undried portion and the mixture

[1] Purchased from Ross Laboratories.

kept at 0°C until the lactose content of a filtered sample reached a constant minimum value. The lactose precipitate was removed by filtration through a Buchner funnel and washed with 2 × 200 ml of ice-cold water. The filtrate and washings were combined and lyophilized. Three volumes of acetone were stirred into the cream and the precipitate which formed was removed by centrifugation, washed three times with a volume of acetone equal to the volume of the precipitate and dried at 50°C overnight in vacuo. The fractions were combined, thoroughly mixed and passed through a 20-mesh

TABLE 1

Analysis of lyophilized cow's and human milk protein preparations

Experiment 1

ANALYSIS	HUMAN MILK CONCENTRATE	LYOPHILIZED SKIM COW'S MILK
	%	%
Total nitrogen	3.26	5.68
Protein nitrogen	2.65	5.42
Non-protein nitrogen [1]	0.602	0.262
NPN as % of total N	18.5	4.6
Protein (N × 6.38)	20.8	36.2
Fat	4.68	1.38
Water	5.55	4.00
Ash	3.65	8.00
Lactose [2]	65.3	52.0

[1] Tungstic acid filtrate.

[2] Sumner's method ('25).

screen. The final product contained 95% of the nitrogen, 49% of the lactose, and 7% of the fat contained in the original human milk sample.

The cow's milk preparation was a sample of a large composite of skim milk, pasteurized at 140°F for 30 minutes. The skim milk was lyophilized and stored in the cold under nitrogen until use.

Analytical data for the two milk protein samples are presented in table 1.

Experiment 2. Three and one-half liters of mature human milk,[2] 161 mg N/100 ml, was skimmed as described above, dialyzed for 48 hours against cold running tap water, and lyophilized; 44 gm of solids containing 9.26% nitrogen were obtained.

Approximately two liters of skim cow's milk was treated similarly; 59 gm, 12.5% nitrogen, were recovered.

One dozen boiled fresh eggs were shelled, extracted with 750 ml of acetone 4 times in a Waring blendor, and then twice with an equal volume of ethyl ether. The residue was dried overnight at 40°C in a vacuum oven; yield, 103 gm, 12.2% nitrogen.

Bioassays. The following methods were used for the determination of the nutritive value of the protein:

$$\text{Protein efficiency ratio (PER)} = \frac{\text{weight gain}}{\text{protein consumed}} \qquad \text{(a)}$$

$$\text{Net protein utilization (NPU)} = \frac{\text{carcass N increase} + \text{non-protein carcass N loss}}{\text{food N}} \times 100 \qquad \text{(b)}$$

(Miller and Bender, '55)

$$\text{Protein retention efficiency (PRE)} = \frac{\text{wt. gain} + \text{wt. loss of non-protein group}}{\text{protein consumed}} \times 16 \qquad \text{(c)}$$

(Bender and Doell, '57)

$$\text{Biological value (BV)} = \frac{\text{food N} - (\text{fecal N} - \text{metabolic N}) - (\text{urine N} - \text{endogenous N})}{\text{food N} - (\text{fecal N} - \text{metabolic N})} \times 100 \qquad \text{(d)}$$

(Mitchell, '23–'24)

$$\text{True digestibility (TD)} = \frac{\text{food N} - (\text{fecal N} - \text{metabolic N})}{\text{food N}} \qquad \text{(e)}$$

$$\text{Net protein utilization (NPU)} = \text{biological value} \times \text{true digestibility} \qquad \text{(f)}$$

Water and nitrogen determination. The carcass of the animal, partially skinned and with the viscera exposed, was dried in vacuo at 100°C for 48 hrs. in a shallow aluminum foil dish covered with a sheet of filter paper to prevent loss of fat. The dish plus contents were weighed for determination of water loss. The dried brittle carcass and the filter paper cover were cut into small pieces, mixed with an equal weight

[2] Kindly supplied by Dr. Paul György.

of powdered cellulose [3] and ground in a plate grinder. The cellulose absorbed the fat, facilitated the grinding, and resulted in a more homogenous sample for nitrogen analysis.

RESULTS

Experiment 1. The nutritive values of the protein of skim cow's milk and of the human milk concentrate of reduced lactose content were determined in a 28-day rat growth test in which the protein preparations were fed a 10% level (1.57% N × 6.38). A control diet was included in the test to compen-

TABLE 2

Composition of diets

Experiment 1

INGREDIENT	DIETS				
	A	B	C	D	E
Human milk concentrate	482.0	—	—	—	—
Cow's milk	—	276.0	236.0	—	—
Casein	—	—	—	—	240
Lactose	—	171.2	192.0	314.7	320
Lard	177.4	196.2	196.7	200.0	200
Salts [1]	12.4	17.9	21.1	40.0	40
Glucose	345.3	345.3	354.3	445.3	200
Urea-glutamic acid mixture [2]	—	—	7.44	—	—

[1] Hubbell et al. ('37).

[2] Three parts urea and one part glutamic acid.

sate for the difference in non-protein nitrogen of human and cow's milk; the analytical data of table 1 show the magnitude of this difference. The compositions of the diets are presented in table 2.

Each kilogram of diet was fortified with 1 gm of choline chloride, 5 mg each of thiamine·HCl, riboflavin, pyridoxine·HCl and 2-methylnaphthoquinone; 50 mg each of niacin and Ca pantothenate; 100 mg each of inositol and *p*-aminobenzoic acid; 2 mg of folic acid, 0.5 mg of biotin and 50 μg of vitamin B_{12}. Fat soluble vitamins were given by dropper, 2 drops

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

twice weekly: 30 mg of mixed tocopherols, 4400 units of vitamin A and 600 units of vitamin D per gram of corn oil.

Each test diet contained per kilogram, 100.0 gm protein, 315 gm lactose, 200 gm of lard, 40 gm of salt, and the remainder glucose. Allowance was made for the lactose, fat and ash content of the protein preparations. All the diets were isonitrogenous, isocaloric, and had the same lactose content. Diet C, containing the same amount of protein as diet A, was supplemented with a mixture of three parts of urea, one part glutamic acid to simulate the non-protein nitrogen fraction of

TABLE 3

Protein retention efficiency of human and cow's milk [1]

Experiment 1

GROUP	SOURCE OF PROTEIN	PROTEIN N AS % OF TOTAL N	WEIGHT GAIN OVER D ± STANDARD ERROR	PROTEIN EATEN [2]	PROTEIN RETENTION EFFICIENCY [3]
			gm	*gm*	
A	Human milk	81.5	56 ± 3.3	14.8	60.5
B	Cow's milk	95.4	59 ± 2.9	15.7	60.0
C	Cow's milk	81.5	54 ± 2.3	13.9	62.1
D	(Protein-free)		(— 23 ± 1.7)		

[1] Statistical significance of weight gain differences. Student's "t" test: A vs. B, p = 0.5; B vs. C, p = 0.2; A vs. C, p = 0.6.

[2] Protein N × 6.38.

[3] Fourteen-day test (Bender and Doell, '57).

human milk. Nitrogen analyses were run on the completed diets to verify the expected protein content.

Forty-five male weanling Sprague-Dawley rats individually caged in an air-conditioned room were fed diet E of table 2 for 10 days to let them become partially adapted to the lactose diet. Thitry-two of the more typical rats on the basis of weight gain were divided by weight into 4 groups of 8 rats each; diets A, B or C of table 2 were fed to three of the groups for 28 days; diet D, the protein-free diet, was fed for 14 days. Food and water were given ad libitum. Food consumption of each group was recorded.

The results obtained after 14 days, calculated for protein retention efficiency, are presented in table 3.

The calculation of protein retention efficiency indicates no difference in the nutritional value of the two milk proteins. As expected, group B, fed a diet higher in protein than group A or C, 95 vs. 82% of the total nitrogen, gained slightly more weight. This difference was not of statistical significance. The protein retention efficiency values for both proteins are low presumably because of the growth inhibitory effect of lactose.

TABLE 4

Protein efficiency ratio of human and cow's milk

Experiment 1

Weight gain and protein efficiency ratio [1,2]

GROUP	PROTEIN [3]	PROTEIN N AS % OF TOTAL N	14 DAYS		28 DAYS	
			Gain	PER [1]	Gain	PER [1]
			gm		*gm*	
A	Human milk	81.5	33 ± 2.9	2.23	70 ± 3.6	2.38
B	Cow's milk	95.4	36 ± 2.4	2.29	93 ± 3.3	2.60
C	Cow's milk	81.5	31 ± 1.6	2.23	78 ± 1.5	2.55

[1] Grams gain per gram protein eaten; protein = N × 6.38.

[2] Statistical significance of weight gains.

Student's "t" test probability
(p = 0.05 or less considered significant.)

	14 DAYS	28 DAYS
A vs. B	0.4	< 0.001
A vs. C	0.5–0.6	0.07
B vs. C	0.1–0.2	< 0.001

[3] All diets isonitrogenous.

The average weight gains and protein efficiency ratios of the three groups of rats at 14 and 28 days are presented in table 4. Group B, fed cow's milk protein at a higher level, attained a greater body weight than the other two groups; the differences in weight gains were highly significant at 28 days. A comparison of the proteins fed in diets of equal protein content, groups A and C, indicated no difference in nutritional value. The rats in the human milk group were slightly heavier than those in the cow's milk group at 14 days and slightly

lighter at the 28th day. The weight gain differences were not statistically significant.

Experiment 2. In this experiment the nutritive values of cow's and human milk protein were compared on protein freed of lactose by dialysis. Only 3% of the total amino acid N in human milk and 0.8% in cow's milk are in the non-protein fraction (Macy et al., '53); furthermore Block and Bolling ('50) have found that the amino acid pattern in the non-protein fraction is essentially similar to that in the protein fraction in both human and cow's milk. Whole egg protein was also assayed to permit a comparison of the milk proteins with a protein known to be almost completely utilized by the growing rat.

The diets contained sufficient protein to give 1.25% of nitrogen (8% protein), 15% lard, 4% salts, vitamin mixture as in experiment 1 and the balance glucose. Fat-soluble vitamins were given by dropper.

Young male Sprague-Dawley rats, divided by weight into groups of average weight of 82 gm, were housed in individual metabolism cages in a constant temperature room and given food and water ad libitum. Individual diet consumptions were recorded to permit statistical evaluation of the protein efficiency ratio and protein retention efficiency. The rats were permitted a two-day acclimation period in the metabolism cages and the feces and urine were collected daily for the next 5 days. One milliliter of 6 N hydrochloric acid was placed in the urine collection flasks and urine and feces samples were kept frozen until analysis.

Nitrogen and water contents of the carcasses, presented in the data of table 5, are in agreement with the observation of Miller and Bender ('55) that the nitrogen-water ratio is a constant at any given age and permits a calculation of carcass nitrogen from the water content. Calculation of nitrogen from the water content data of table 5 using the Miller-Bender equation yielded values that were 2 to 6% lower than the Kjeldahl values but the exact age of the rats was unknown; it

was assumed that they were 24 days old when received from the supplier.

The various nutritive indices were calculated from individual data. Corrections for maintenance nitrogen, metabolic nitrogen and endogenous nitrogen were the average figures for the non-protein group.

The calculation of protein efficiency ratio, protein retention efficiency and net protein utilization revealed no real differences between human and cow's milk protein, table 6. The

TABLE 5

Diet and carcass content of nitrogen and water[1,2]

	DIET	NITROGEN ± S.E.	WATER ± S.E.	N: H_2O ± S.E.
		%	%	%
A	Protein free	2.94 ± 0.04	70.4 ± 0.5	4.17 ± 0.08
B	Egg	2.78 ± 0.04	69.4 ± 0.5	4.00 ± 0.02
C	Human milk	2.81 ± 0.04	69.5 ± 0.6	4.05 ± 0.07
D	Cow's milk	2.85 ± 0.02	69.1 ± 0.5	4.13 ± 0.03

[1] Sprague-Dawley rats approximately 36 days old.
[2] Statistical probability by "t" test.

GROUPS	N	H_2O	N/H_2O
A vs. B	0.02	0.2	0.05
A vs. C	0.05	0.3	0.8
A vs. D	0.07	0.1	0.6

slightly higher values obtained for cow's milk protein were not statistically significant, ($p = 0.6$, 0.6 and 0.4 respectively).

The calculation of protein retention efficiency gives a numerical value that agrees with that of net protein utilization (Bender and Doell, '57). In the present experiment the net protein utilization values are obviously high, namely, egg protein 109. When the values for human and cow's milk are recalculated based on a 96.4% utilization of egg protein, net protein utilization values of 75 and 78 are obtained which agree well with the protein retention efficiency values of 74 and 77.

TABLE 6

Bioassay of egg, human milk and cow's milk protein

Experiment 2

GROUP	WEIGHT GAIN	FOOD N	PROTEIN EFFICIENCY RATIO	PROTEIN RETENTION EFFICIENCY	CARCASS N	CARCASS N INCREASE	NET PROTEIN UTILIZATION CARCASS N METHOD
	gm	*mg*	*gm/gm*		%	*mg*	
Protein-free	— 8.9				3.00	— 267	
	— 8.5				2.86	— 243	
	— 7.8				3.04	— 237	
	— 8.6				2.95	— 254	
	— 7.9				2.82	— 223	
Av.	— 8.3				2.94 ± 0.04	— 244	
Egg	20.5	769	4.2	93.9	2.77	566	106.0
	19.5	709	4.3	98.4	2.87	542	113.8
	22.1	780	4.4	97.6	2.66	614	107.0
	17.1	687	3.9	92.8	2.75	475	104.0
	21.6	755	4.5	99.2	2.83	600	113.6
Av.	20.2		4.3 ± 0.1	96.4 ± 1.3	2.78 ± 0.04		108.9 ± 2.0
Human milk	9.4	678	2.2	65.4	2.87	270	76.3
	13.0	724	2.8	73.8	2.88	374	85.8
	11.8	691	2.7	73.0	2.75	325	82.8
	15.6	713	3.4	84.0	2.75	429	94.8
Av.	12.5		2.8 ± 0.3	74.1 ± 3.6	2.81 ± 0.04		84.9 ± 3.8
Cow's milk	16.8	814	3.2	77.4	2.78	467	87.7
	7.9	486	2.6	83.7	2.91	230	98.1
	16.3	779	3.2	79.2	2.88	469	91.9
	16.9	800	3.3	79.0	2.85	482	91.1
	10.8	723	2.3	65.9	2.83	306	76.5
Av.	13.7		2.9 ± 0.2	77.0 ± 3.0	2.85 ± 0.02		89.1 ± 2.3

The data required for the calculation of biological value are presented in table 7. Human milk protein had a slightly higher biological value than the cow's milk protein but the net utilization was slightly lower because of the poorer digestion coefficient. The differences in the three indices were not of statistical significance, $p = 0.7$, 0.07 and 0.9 for biological value, true digestibility and net protein utilization respectively.

DISCUSSION

Little difference in the nutritional quality of the protein of human and cow's milk has been demonstrated in these bioassays based on the utilization of protein by a third mammalian species. Cow's milk protein appeared slightly better in tests measuring the overall utilization of protein, a superiority which appears to result from a more complete absorption of the nitrogen of cow's milk protein, since the biological value of human milk protein was slightly higher than that of cow's milk. None of the differences in nutritional indices were of statistical significance, though the lower true digestibility of human milk compared to cow's milk approached significance. There is experimental evidence that human milk protein is more resistant to proteolysis than cow's milk protein (Kennedy et al., '55), and while this may afford a nutritional advantage to the infant (Mellander and Vahlquist, '57), it may result in incomplete absorption for the rat.

A rat bioassay of protein quality is a measure of the availability of the limiting essential amino acid in the protein tested. The sulfur-contaning amino acids are limiting in milk protein (Mitchell and Block, '46), and it is evident from the present study that the combination of methionine and cystine in human milk protein is nutritionally equivalent to that in cow's milk for the growing rat. This might have been expected from present knowledge of the methionine-cystine relationship in rat metabolism and from the analytical data on the milk proteins. Cow's milk protein contains 2.6% of methionine and 0.9% of cystine (Macy et al., '53); human milk protein,

TABLE 7

Bioassay of egg, human milk and cow's milk protein

Experiment 2

GROUP	FOOD N	FECAL N	URINE N	BIOLOGICAL VALUE	TRUE DIGESTIBILITY	NET PROTEIN UTILIZATION
	mg	*mg*	*mg*		%	$BV \times TD$
Protein-free		43.4	80.0			
		23.1	92.3			
		28.5	84.2			
		26.7	96.5			
		34.4	71.5			
Av.		33.2	84.9			
Egg	769	72.5	89.1	99.4	94.9	94.3
	707	52.9	75.7	101.3	97.2	98.5
	780	65.1	96.9	98.4	95.9	94.4
	687	66.6	86.3	99.8	95.1	94.9
	755	61.9	96.2	98.4	96.2	94.7
Av.	740	63.8	88.8	99.6 ± 0.5	95.9 ± 0.4	95.4 ± 0.8
Human milk	678	88.6	120.1	94.3	91.8	86.5
	724	132.5	135.3	91.9	86.3	79.3
	691	60.7	142.0	91.4	96.0	87.7
	713	123.4	155.7	88.6	87.3	77.3
Av.	702	101.3	138.2	91.6 ± 1.2	90.4 ± 2.2	82.7 ± 2.6
Cow's milk	814	74.4	217.1	95.8	94.9	90.9
	486	50.2	101.5	75.1	96.5	72.5
	779	83.7	155.6	89.6	93.5	83.7
	800	76.6	157.5	90.4	94.6	85.5
	723	72.2	147.7	98.1	94.6	92.8
Av.	720	71.4	171.9	89.8 ± 4.0	94.8 ± 0.5	85.1 ± 3.6

2.0% of methionine and 2.3% of cystine (Soupart et al., '54). Thus the total amount of sulfur-containing amino acids is greater in human milk than in cow's milk but the pertinent point is whether the excess cystine can compensate for the lower concentration of the essential methionine. Wretlind and Rose ('50) in studies with the weanling rat found that the methionine requirement is 0.3 to 0.4% with a complete diet containing 0.2% of cystine; i.e., a methionine-cystine mixture of 1 to 0.7 or 0.5 is as effective as methionine alone. In a study, more comparable to that of the present publication in that diets containing 8% of whole milk protein were fed, Henry and Kon ('53) found a maximal biological value with a diet fortified to contain 0.22% of methionine plus 0.15% of cystine, a methionine-cystine ratio of 1 to 0.7. Egg protein, which is most efficiently utilized, contains a methionine-cystine ratio again of 1 to 0.6. Assuming that cystine can be effectively utilized in an amount equivalent to 0.6 that of the methionine content, cow's milk protein would have a methionine equivalent of 3.5%, (2.6 + 0.9%) and human milk 3.2%, (2.0 + 0.6 of 2.0%). The assumption that not all the cystine of human milk is utilized would appear to be justified by the present finding that human milk and cow's milk protein are of equal nutritional value for the rat. The extent to which cystine can substitute for methionine in the protein nutrition of the infant is not known but the question is almost academic since there can be little question that the ideal protein composition to strive for in the preparation of an artificial formula is that of human milk.

SUMMARY

Biological assays of the protein of human and cow's milk showed little difference in the nutritional value in experiments designed to reduce excessive amounts of dietary lactose. The lactose of human milk was reduced by crystallization in the one assay and by dialysis in the second. There was no difference of statistical significance in the nutritional indices of protein efficiency ratio, protein retention efficiency, net pro-

tein utilization (by carcass N analysis), biological value, true digestibility and net protein utilization (biologcal value × true digestibility).

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THE UTILIZATION OF DIETARY PROTEIN AND ENERGY AS AFFECTED BY FAT AND CARBOHYDRATE [1,2]

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In an extensive series of studies with albino rats it was shown that a diet high in fat was more efficiently utilized than was a similar diet low in fat furnishing an equal and adequate amount of protein and energy (Forbes and associates, '46a, b, c, d; Black et al., '49 a, b; Swift and Black, '49). Using diets containing from 2 to 30% of fat it was found with both growing and mature animals that a consistent decrease in heat production took place with each increase of the dietary fat level. French et al. ('48) on feeding equicaloric diets containing 2 and 30% fat, respectively, but restricted in protein, also found that superior energy utilization, increased body gains of fat and energy, and decreased heat production were associated with the high-fat diet.

EXPERIMENTAL

The work reported here was planned to determine whether these findings, well established with albino rats, are also

[1] Authorized on November 7, 1958 for publication as paper no. 2310 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Department of Animal Nutrition Publication no. 243, College of Home Economics Research Publication 167.

[2] This study was part of a Northeast Regional Project (NE-37, Relationships between protein and other selected nutrients and their metabolism and utilization); a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds.

applicable to humans. Two diets, markedly different in fat content, were prepared to furnish equal amounts of protein and energy daily. The diets were analyzed before the experiment began and provided 3035 Cal. and 97.5 gm of protein daily. The percentages of fat in the two diets, on the dry matter basis, were 4.86 and 34.50 respectively. The percentages of the total energy derived from fat in the low-fat and in the high-fat diets were 9.8 and 60.1, respectively. As calculated, the intake of all other nutrients exceeded the National Research Council Allowances ('58).

The main food items of the two diets were the same (table 1).

Because of the low-fat nature of the basal diet, ground veal rather than ground beef was used. The low-fat diet was composed of the basal diet supplemented with high carbohydrate foods. Grape jelly, sucrose, and an unsweetened grapefruit juice-cerelose mixture (2.46 gm grapefruit juice to 1 gm cerelose) provided the extra energy for the low-fat diet. Being less sweet, cerelose was used rather than sucrose. The food supplements of the high-fat diet were butter and heavy cream.

In this study, the foods of the experimental diets were purchased, stored, prepared, and sampled for analyses in the same manner as reported in a previous investigation (Swift et al., '58). All the foods of the diets were weighed to the nearest gram. After each subject had eaten the food served to him, bread was used to wipe the last traces from the plate. To minimize any possible undesirable psychological responses, the subjects on the low-and high-fat diets were served in separate dining rooms.

Two pairs of male subjects, about 23 years of age were selected on the basis of uniform weight (154 lbs.), basal metabolism and interest in the project. Each of the two subjects of a pair received identical diets during a given period. In all cases, the first calorimeter period took place three days after the subjects had been assigned to a particular diet. Both subjects of a pair were in the respiration calorimeter at the same time for the 24-hour measurements of heat (in-

TABLE 1

Experimental diets

MEAL	MAIN FOOD ITEMS	SUPPLEMENTARY FOODS	
		High-fat	Low-fat
	gm	*gm*	*gm*
Breakfast	Oatmeal (dry), 40 Skim milk, 246 White bread, 46	Orange juice, 100 Sucrose, 20 Butter, 30 Heavy cream, 79	Orange juice, 246 Sucrose, 40 Jelly, 35
Lunch	Ground veal, 100 Green beans, 100 Lettuce, 50 Skim milk, 246 White bread, 46	Peaches, 100 Butter, 33 Heavy cream, 79	Peaches, 200 Jelly, 35 Grapefruit juice-cerelose mix,[1] 357
Dinner	Ground veal, 100 Carrots, 100 Celery, 100 Skim milk, 246 White bread, 46	Apricots, 100 Butter, 30 Heavy cream, 79	Apricots, 200 Jelly, 30 Grapefruit juice-cerelose mix,[1] 357

[1] Mix — 123 parts (by weight) grapefruit juice + 50 parts cerelose.

cluding water vaporization) and carbon dioxide. With the subjects continuing on the same diet, the calorimeter period was repeated one week later.

To provide the data for the determination of the nitrogen balance, 5-day collection periods (feces and urine) for each individual took place between the two consecutive calorimeter periods representing a given diet.

One pair of subjects received the low-fat diet first with subsequent assignment to the companion diet high in fat. The reverse order was followed with the other pair. The average weight of the subjects at the end of the experiment was practically the same as at the beginning. One pair lost a total weight of one pound; the second pair, one-half pound.

The preliminary period of 6 hours in the respiration calorimeter and other details of operation and control were the same as were described in a recent publication (Swift et al., '58). A limited amount of prescribed exercise was performed by the subjects while in the calorimeter in an attempt to make equal the amounts of activity involved in each calorimeter period. The subjects entered the chamber several times for a few hours prior to the actual test to become accustomed to the routine. The usual preliminary tests were made to verify the tightness of the chamber and ventilation lines and to recover quantitatively a known amount of CO_2 which was released into the calorimeter.

DISCUSSION OF RESULTS

As indicated in table 2, the 24-hour metabolism of each pair of subjects was measured twice during a given dietary regime, these measurements being one week apart. In previous work (Swift et al., '58), it was found that the 24-hour metabolism was the same at the beginning of the dietary regimes under study as it was when measured at intervals up to 7 weeks later. In the present work, however, as shown in table 2, with both pairs of subjects on the low-fat diet, the production of heat and carbon dioxide was about 3% greater on the 10th day than on the third day. This increase in metabolism is not very

great but it suggests the possibility, in accord with the extensive work with albino rats, that a longer regime on the low-fat diet with humans might have revealed a consistently higher heat loss. Some support for this possibility is also indicated by the extremely close agreement between duplicate periods on the high-fat diet with both pairs of subjects.

A comparison of the two diets, using all the data from the 8 periods of observation, shows that the average daily heat production resulting from the low-fat diet was 4990 Cal. as compared to 4993 Cal. from the high-fat diet.

A comparison of the two pairs of subjects shows that one pair of subjects produced 3.2% more heat while on the low-fat diet than when receiving the high-fat diet. The same comparison with the other pair shows a difference of 3.3% in the opposite direction. Under the conditions of this experiment with limited opportunity for adaptation to be influential, it seems apparent that there is no appreciable difference in the energy utilization by humans of equicaloric diets containing widely different amounts of fat.

In the series of studies with albino rats previously referred to, the differences in body gain and heat production obtained on comparing diets low or high in fat were associated, not with differences in metabolizable energy but rather, with the division of this item into heat production and body gain. The same observation would seem to apply to the present work with humans regarding the small increase noted in the second period of heat measurement on the low-fat diet. The differences between the two diets in terms of digestible energy and metabolizable energy were less than one per cent.

As would be expected, the CO_2 production was definitely less when the subjects were consuming the diet high in fat. The non-protein respiratory quotients (table 2) were calculated from the urinary nitrogen, the total heat and the total CO_2 production.

The average daily nitrogen balance for subjects C. R. and J. S. on the low-fat diet was + 0.27 gm; on the high-fat diet, +1.91 gm. Corresponding values obtained with the other

TABLE 2

Daily heat and CO_2 production of pairs of subjects on equicaloric diets

	Days on diet	Period no.	CO_2	Non-protein R. Q.	Heat production	
					Total Cal.	As water vapor
SUBJECTS C. R. AND J. S.			*liters*			%
Low-fat diet	3	1	913.7	0.90	5003	26.9
	10	2	950.8	0.92	5146	29.1
High-fat diet	3	5	812.9	0.76	4925	30.7
	10	6	789.4	0.76	4915	32.0
SUBJECTS F. K. AND R. T.			*liters*			%
High-fat diet	3	3	803.1	0.75	5062	30.1
	10	4	800.2	0.71	5068	31.3
Low-fat diet	3	7	892.9	0.92	4837	29.8
	10	8	907.8	0.90	4973	31.6

two subjects were + 3.21 and + 0.42 gm, respectively. In view of the fact that the subjects were on the rigidly controlled diets for periods of only 10 days, it does not seem permissible to ascribe any significance to the difference observed in nitrogen balances. Positive nitrogen balances in all cases however indicate that the protein intake was adequate and was not needed as a source of energy.

With respect to the manner of heat disposal, there seems to be no appreciable difference in the percentages of the total heat which was eliminated by the evaporation of water as affected by the composition of the diets. The average of all periods (30.2%) is closely similar to the previous value obtained (31.0%) in comparable work involving high- and low-protein diets (Swift et al., '58).

SUMMARY

The 24-hour metabolism of two pairs of male subjects, about 23 years of age, was measured by use of the respiration calorimeter. Two diets furnishing equal amounts of protein and energy but differing widely in fat content were compared as to daily nitrogen balance and heat production. One pair of subjects received the low-fat diet first with subsequent assignment to the companion diet high in fat. The reverse order was followed with the other pair.

The differences in total daily energy expenditures and nitrogen balances between the two diets were insignificant.

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SALT MIXTURES FOR PURIFIED-TYPE DIETS

II. EFFECT OF SALTS ON THE MAILLARD BROWNING REACTION

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During a study of oxidative rancidity in purified diets (Fox and Mickelsen, '59), it was observed that certain diets became brown within a few days. The formation of the brown-colored material was found to be the type of browning first described by Maillard ('12) in which free amino groups of proteins or amino acids react with a reducing sugar to form a great variety of products. Many workers have reported detrimental effects of these reactions on the nutritional value of protein sources and other important nutrients containing an amino group, such as thiamine (see review by Patton, '50). The complexity of the reactions and the diversity of the products formed have been reviewed (Danehy and Pigman, '51; Hodge, '53). The rate of diet-browning in the present experiments was markedly accelerated by certain salt mixtures. Identification of the salt responsible for the increase in browning was made and the effects of certain conditions important to the formulation and use of experimental diets for animals were also studied in relation to the browning reaction.

EXPERIMENTAL PROCEDURE

Chick diet C2 (Fox, Ortiz and Briggs, '55), a complete purified diet in which the protein need was supplied by casein, gelatin, and methionine and the carbohydrate by glu-

cose, was used in the first experiments. This diet was prepared with three different salt mixtures, Chick Salts A (Briggs et al., '52), HMW Salts (Hubbell, Mendel, and Wakeman, '37), and Wesson Salts (Wesson, '32). In most experiments, the effect of individual salts and other dietary components upon the browning reaction was determined in a simple mixture of crude glucose[1] and glycine. Since this mixture was white, small changes in color were readily apparent; furthermore, the mixture was restricted to the components responsible for browning. The samples were mixed in a laboratory in which the relative humidity ranged between 40 and 50%. Samples were stored in tightly closed clear glass vials about three-fourths full. Storage temperatures and duration of experiments are indicated in the tables. Samples under each storage condition were scored daily against each other and rated on a scale from 0, representing no color change, to 5, representing considerable browning.

RESULTS

In the preliminary experiments it was found that the presence of Chick Salts A in the complete diet stored at 37°C resulted in the formation of a tan color flecked with brown dots after a few days. When the same diet was prepared with either Wesson or HMW Salts in place of Salts A, very little change in color was seen after three weeks of storage. The effects of these salts upon browning of the complete diet were reproduced in a mixture of glucose and glycine; agreement was very close between duplicate experiments. The results were the same with either reagent grade or crude glucose, but no browning was observed when sucrose replaced the glucose.

The major mineral constituents of Chick Salts A ($CaCO_3$, $Ca_3(PO_4)_2$, K_2HPO_4, Na_2HPO_4, and $MgSO_4$) were combined in one mixture, Salts A-1, while the "trace" minerals were combined in another mixture, Salts A-2. Browning occurred

[1] Cerelose.

only with Salts A-1. When the individual salts in mixture A-1 were tested, only K_2HPO_4 accelerated the rate of browning at 37°C. A combination of all constituents of Salts A-1 other than K_2HPO_4 produced no browning and the browning produced by K_2HPO_4 alone was equal to that of the complete Salts A-1. Ten commercial salt mixtures commonly used in animal diets have been checked under these conditions and only those containing K_2HPO_4 caused browning.[2]

TABLE 1

Effect of temperature, moisture, and several common dietary salts on the browning of glycine and glucose[1]

SALT ADDED	WEIGHT PER SAMPLE	BROWNING[2] AT		
		37°C	37°C 2.5% Water added	50°C
	gm			
None	0	0	1	1
$Ca_3(PO_4)_2$	0.14	0	1	3
$CaCO_3$	.14	0	1	3
Na_2HPO_4	.09	0	5	5
K_2HPO_4	.09	5	5	5
$MgSO_4$	.01	0	1	2

[1] Average of three experiments. Each mix contained 8 gm crude glucose and 1 gm glycine; salts were incorporated at concentrations similar to those supplied in a diet by Chick Salts A at a level of 6%. All samples were tightly capped.

[2] Samples stored at 37°C were scored at 28 days; a score of 5 represented tan with dark brown granules. Samples with added water stored at 37°C were scored at 6 days; 5 represented dark brown. Samples stored at 50°C were scored at three days; 5 represented black, charred, and shrunken. At each temperature one was a very light tan.

The effect of the constituents in Salts A-1 upon glucose-glycine browning at 37°, at 37° with 2.5% additional water in the mixture, and at 50°C may be seen in table 1. The addition of water to the mixture caused equal browning to occur with Na_2HPO_4 and K_2HPO_4 at 37°C. Moisture itself caused some browning to occur in the mixture of glucose and glycine. At 50°C, the mixtures containing Na_2HPO_4 or K_2HPO_4 were

[3] Browning occurred with Salt Mixture No. 2 U.S.P. XIII, Salt Mixture U.S.P. XIV, and Salt Mixture P-H. These were all purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

black by the end of three days. Browning greater than that of the control was also seen in mixtures containing each of the other three salts that were tested.

The influence of glycerol on the above reactions was studied because Waibel, Bird and Baumann ('54) found that the destructive effect of K_2HPO_4 upon thiamine in purified diets could be reduced by adding glycerol at a level of 1%. In almost all samples that eventually browned (table 2), the formation of a brown color was detectable after 24 hours. The K_2HPO_4 again caused browning to be more severe, even under conditions of minimal moisture content (sample 9). Glycerol caused considerable browning of the mixture of glycine and glucose under each storage condition (sample 11); however, glycerol did not affect the browning caused by K_2HPO_4 (sample 16). Moisture and especially the combination of moisture and higher storage temperatures increased the browning rate. As a closer approach to actual animal feeding conditions, the vials stored at 25°C were left uncovered in an animal room. Samples that browned at 37° and 50°C also browned at 25°C.

DISCUSSION

The desirability of keeping the Maillard browning reaction at a minimum in experimental diets is well known. In diets containing free amino acids, the number of available reactive groups is greatly increased so that browning may occur much more rapidly and extensively than in protein-containing diets. The problem is particularly critical in diets with a borderline level of an essential amino acid.

The activity of K_2HPO_4 in promoting the browning reaction appears to reside in the compound's basicity and hygroscopic nature. The amount of browning has long been known to increase with increasing pH (Danehy and Pigman, '51). Dibasic sodium phosphate, which is equally basic but less hygroscopic, was not equally destructive until the moisture content of the mixture was increased. Because of the

TABLE 2

Effect of temperature, K_2HPO_4, glycerol, and water on the browning of glycine and glucose

CONSTITUENT	GM PER SAMPLE	SAMPLE NUMBER															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Crude glucose	9	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Glycine	1		X	X	X					X	X	X	X			X	X
K_2HPO_4	0.1					X	X			X	X			X	X	X	X
Glycerol	0.1							X	X			X	X	X	X	X	X
Water	1.0				X		X		X		X		X		X		X
Browning [2] at	25°C	0	0	0	3	0	0	0	0	4	5	2	3	1	0	4	5
	37°C	0	0	0	4	0	0	0	0	3	5	2	3	1	0	3	5
	50°C	0	0	0	5	0	1	0	0	2	5	3	5	1	1	3	5

[1] Each X indicates the presence of that constituent in a mixture. Average of two experiments. Glucose, glycine, and K_2HPO_4 dried to constant weight at 70°C under vacuum. Samples at 37° and 50°C stored in tightly capped vials; samples at 25°C stored in open vials.

[2] Samples stored at 25°C were scored at 28 days; a score of 5 represented medium brown. Samples stored at 37°C scored at 28 days; 5 represented black. Samples stored at 50°C were scored at three days; 5 represented black, charred, and shrunken. At each temperature, one was a very light tan.

effect upon both browning and destruction of thiamine, it appears advisable to avoid using salt mixtures that contain K_2HPO_4 in experimental diets. Equivalent amounts of potassium and phosphorus can be supplied by other salts. The importance of low moisture content and low storage temperatures in suppressing browning have again been emphasized. When it is necessary to store diets even for short periods of time at high temperatures with or without high moisture content, salts other than K_2HPO_4 may markedly increase browning. Under such severe storage conditions, it might be advisable to omit one type of compound involved in the browning reaction (reducing sugar, amino compounds, or salts) during the storage period and then add that compound just prior to feeding.

SUMMARY

Purified diets containing glucose and protein developed a brown discoloration during storage when Chick Salts A was present. The browning reaction also occurred in a mixture of glycine, glucose, and Salts A. The effect of the ingredients of the salt mixture in this simplified system was studied and K_2HPO_4 was found to be by far the most active salt in the promotion of this reaction. Increases in moisture and in storage temperature of experimental mixes markedly increased the amount of browning that occurred in a given period of time. Salt mixes such as Wesson and Hubbell, Mendel and Wakeman, which contained no K_2HPO_4, had no effect on the browning reaction under ordinary conditions of diet mixing and storage.

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THE UPTAKE AND TURNOVER OF RADIOACTIVE VITAMIN B_{12} IN RABBIT TISSUES [1]

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The concentration of vitamin B_{12} in rabbit blood plasma (Couch et al., '50; Ross, '50; Rosenthal and Brown, '54) and urine (Rosenthal and Cravitz, '58) appears to be uniquely greater than that of other experimental animals. Thus, rabbit plasma contains 31 mμg/ml while other mammals such as human, dog and calf contain less than 0.5 mμg/ml (Rosenthal and Brown, '54). Urinary vitamin excretion in rabbits is also very high and averages 375 mμg/kg of body weight per day in animals maintained on diets practically devoid of vitamin B_{12}. This may be compared with urinary vitamin B_{12} excretion of 25 mμg/kg/day for dogs (Rosenthal and Hampton, '55) and 1 mμg/kg/day for human subjects (Register and Sarett, '51; Unglaub et al., '54). On the other hand, the concentration of vitamin B_{12} activity in rabbit tissues and organs appears to be reasonably similar to that of other mammals including man (Lewis et al., '49; Dawbarn and Hine, '54; Shenoy and Ramasarma, '54; Sheid et al., '51; and Swendseid et al., '54). Small differences in tissue concentration and distribution of vitamin B_{12} may be attributed to species differences, analytical procedures, and nutritional factors, but large differences appear to require an explanation of a more fundamental nature (Mil-

[1] A preliminary report of this investigation has appeared (Federation Proc., *17*: 300, 1958).

ler et al., '56). These investigators have shown that the accumulation of injected radioactive vitamin B_{12} is considerably lower in rat kidney than in the kidneys of mice, hamsters and guinea pigs and they suggest that the kidney vitamin B_{12} concentration is independent of the excretory role of the organ. However, the concentration of vitamin B_{12} in r t organs, (especially in the kidney), is not constant, but m e altered by experimental procedures such as starvation (Rosenthal and Cravitz, '58). In rabbits, total starvation for 13 days results in a marked elevation of the vitamin in kidney with smaller increases in liver and heart tissue but plasma and spleen activities remain unaltered. During starvation, urinary vitamin excretion also increases. These changes are largely reversible.

The differences between rabbit plasma and tissue vitamin B_{12} concentration and those of other animals suggest fundamental alterations in the metabolism of this vitamin by the rabbit. In order to study these phenomena more fully, experiments were undertaken to determine the tissue distribution, rate of turnover and excretion of parenterally injected Co^{60} vitamin B_{12} in rabbits. The results of this study form the basis for this report.

MATERIALS AND METHODS

Female virgin New Zealand rabbits weighing 2 to 4 kg were housed in metabolism cages for the collection of urine and feces. The rabbits were fed a commercial rabbit chow except during starvation and water was available to the animals at all times. When coprophagy was prevented, the animals were collared with 10 inch diameter masonite neck pieces. Radioactive Co^{60} vitamin B_{12} of high specific activity (824 mc/gm vitamin B_{12}) was injected via the marginal ear vein or intramuscularly into the left hind leg at a dosage of 0.04 μc/kg of body weight. The animals were sacrificed by air embolism and the tissues were weighed, trimmed of fat and kept frozen until analyzed. Muscle tissue was obtained from the right hind leg and trimmed of fascia and tendons.

The organs, tissues and feces were digested in hot nitric acid and diluted to a minimal volume. Feces extracts were clarified by centrifugation. For estimation of radioactivity, 5-ml aliquots of the digests were counted in a well type scintillation counter. Five-milliliter portions of blood, plasma and urine were counted directly without further treatment. In most instances a sufficient amount of activity was present to yield counting errors of less than 3% but samples of very low activity were counted with an error of less than 10%.

Hemolytic anemia was produced by daily subcutaneous injections of 1 ml of aqueous phenylhydrazine HCl (2.5%) for 4 days prior to the administration of radioactive vitamin B_{12}. After the injection of the vitamin B_{12}, phenylhydrazine was given every other day for an additional week and discontinued thereafter. Hemorrhagic anemia was produced by withdrawing 2% of body weight of blood daily from the heart for 4 days prior to injection of Co^{60} vitamin B_{12}.

RESULTS AND DISCUSSION

Following the intramuscular injection of Co^{60} vitamin B_{12}, tissue distribution of the radioactivity was determined during a 26-day experimental period. The turnover of incorporated radioactivity in liver, kidney, heart, skeletal muscle and brain was negligible during this period and the concentration after 26 days was the same as it was at three days (table 1). However, the radioactivity of spleen tissue and plasma decreased in an exponential manner with a biological half life of 17.3 days for spleen and 4 days for plasma. The radioactivity present in the various tissues three days after injection fall into three main categories. Liver, kidney and spleen accumulate the greatest amounts of the administered dose, while skeletal muscle and brain accumulate the least. Heart tissue assumes an intermediate value. It is of special interest to note that the erythrocytes accumulated negligible amounts of the injected vitamin. With the injection of 0.04 μc/kg of body weight, approximately 4% of the dose was excreted in the urine during the first day, but on succeeding days, the urine

contained negligible amounts of radioactivity. This is in agreement with many other studies in a variety of animals. Samples of feces contained negligible amounts of radioactivity throughout the experimental period. This is in contrast with the recent report by Wider et al. ('58), who showed, in rats, that parenterally injected Co^{60} vitamin B_{12} is excreted in feces. In our experiments, however, the animals were not collared and it was conceivable that the coprophagized soft feces (Kulwich et al., '52; Barnes and Fiala, '58) may have contained some radioactive material.

TABLE 1

Turnover of cobalt-60 vitamin B_{12} in rabbit tissues following intramuscular injection

TISSUE	DAYS AFTER INJECTION			
	3	7	15	26
Liver	10.71 ± 1.23 [1]	13.28 ± 1.16	16.47 ± 1.37	13.52 ± 1.70
Kidney	14.25 ± 3.59	12.67 ± 1.62	14.20 ± 0.80	15.58 ± 2.99
Heart	5.41 ± 0.61	7.16 ± 0.27	4.85 ± 0.73	5.79 ± 0.83
Spleen	12.64 ± 1.92	10.80 ± 1.99	7.88 ± 0.69	4.82 ± 1.36
Muscle	2.76 ± 0.28	3.56 ± 0.11	2.89 ± 0.23	2.79 ± 0.32
Brain	2.45 ± 0.44	2.80 ± 0.59	4.28 ± 0.54	—
Plasma	11.57 ± 2.93	6.53 ± 1.62	3.47 ± 0.74	2.19 ± 0.34
Red cells	0.28 ± 0.18	0.81 ± 0.45	0.25 ± 0.06	0.25 ± 0.24

[1] Mean values ± S.E. determined on 4 to 6 animals. The values are expressed in terms of the percent of the dosage present in 100 gm of tissue. The animals were not collared.

In order to evaluate the factor of coprophagy, animals were collared, injected with Co^{60} vitamin B_{12}, and urine and feces collections were made for 26 days. Except for the urinary excretion of about 3% of the injected dose in the first day's urine, all subsequent urine and feces contained negligible amounts of radioactivity. One possible explanation for this difference may reside in the fact that Wider et al. ('58), used approximately 10 times more vitamin B_{12} per kilogram of body weight than in our experiments. The tissue content of the collared rabbits (table 2) at the end of the experimental period was the same as for the uncollared control animals.

TABLE 2

Distribution of cobalt-60 vitamin B_{12} in tissues of rabbits following various treatments

TREATMENT	LIVER	KIDNEY	HEART	SPLEEN	MUSCLE	BRAIN
			Intramuscular injection			
None, not collared	13.52 ± 1.70 [1]	15.58 ± 2.99	5.79 ± 0.83	4.82 ± 1.36	2.79 ± 0.32	—
None, collared	13.84 ± 1.44	9.65 ± 0.56	5.70 ± 1.12	3.62 ± 1.87	2.78 ± 0.77	2.76 ± 0.50
Anemia, not collared						
Phenylhydrazine	14.35 ± 2.14	8.62 ± 1.46	7.06 ± 0.62	2.95 ± 0.52	1.65 ± 0.16 [2]	—
Hemorrhage	11.29 ± 1.02	12.04 ± 0.90	4.61 ± 0.66	3.47 ± 0.49	2.49 ± 0.10	—
			Intravenous injection			
None, not collared	13.75 ± 0.59	15.36 ± 1.45	6.73 ± 0.60	4.41 ± 1.10	2.40 ± 0.24	2.61 ± 0.77

[1] Mean values ± S.E. determined on 4 to 6 animals 26 days following injection. The values are expressed in terms of percent of the dosage present in 100 gm of tissue.

[2] Significantly different from all other groups at 1% level.

Previous studies show that 60% of the vitamin B_{12} activity in the blood of normal mammals is present in the red cell (Rosenthal and Brown, '54) while Whipple et al. ('55) demonstrated that radioactive vitamin B_{12} injected into anemic dogs was associated with the red cell stroma. As shown in table 1, normal rabbits do not accumulate radioactive vitamin B_{12} in the erythrocytes following an injection of the vitamin. In order to study the incorporation of injected Co^{60} vitamin B_{12} into erythrocytes more fully, rabbits were made anemic with phenylhydrazine or by short term bleeding prior to the injection of the vitamin. The data obtained in these experiments are shown in figure 1. Phenylhydrazine treatment resulted in severe anemia with a reticulocytosis in which 95% of the erythrocytes were relatively young, newly formed cells, indicative of a rapidly proliferating bone marrow. The anemia produced by bleeding was moderate as compared with phenylhydrazine treatment, resulting in a reticulocytosis of 35%. With both types of anemia the hematocrit rose to normal values, and the reticulocyte count fell to base line values within 10 to 12 days after cessation of the anemia-producing regimens.

The anemia produced by phenylhydrazine resulted in a maximum incorporation of 7% of the injected Co^{60} vitamin B_{12} dose/100 ml of red cells, while hemorrhage anemia was associated with the incorporation of 3% of the dose per 100 ml of cells (fig. 1). The largest amount of injected Co^{60} vitamin B_{12} was present in the red cells within two days after dosage. However, the red cell radioactivity rapidly disappeared and after 10 days only negligible amounts could be detected. The rapid decline of erythrocyte radioactivity cannot be explained on the basis of dilution by the increasing hematocrit but appears to be due to loss of radioactivity from the reticulocyte as the cell matures. The transient nature of the incorporated radioactivity in dog reticulocytes has previously been described by Whipple et al. ('55). The distribution of whole blood radioactivity between red cells and plasma reached maximum values within 4 days following the injection of the dose

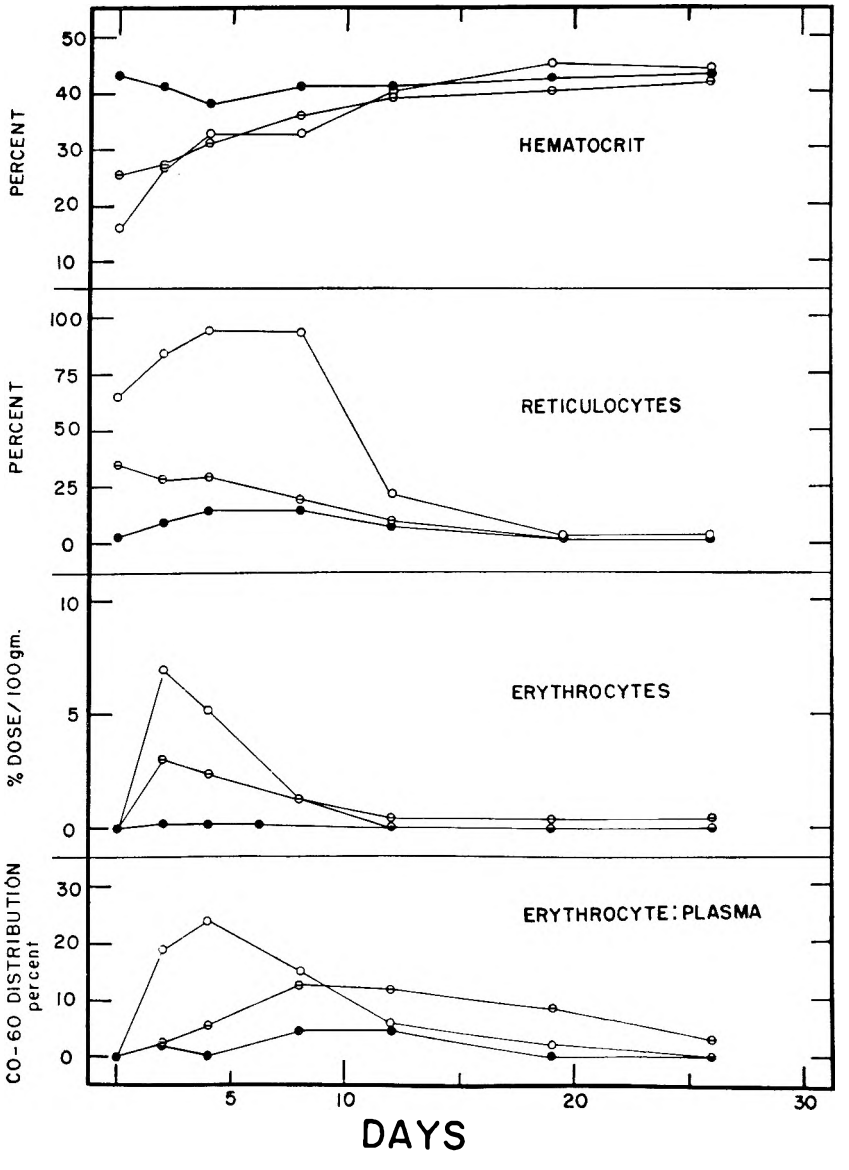

Fig. 1 The hematocrit, reticulocyte count and incorporation of injected Co^{60} vitamin B_{12} in erythrocytes of normal (●), phenylhydrazine anemia (○) and hemorrhage anemia (⊖) rabbits. See text for details.

into the phenylhydrazine-treated animals. This distribution was also transient and fell rapidly as the reticulocytosis decreased. The moderate anemia produced by periodic bleeding resulted in a slower but more prolonged incorporation of radioactivity in the red cells. Adult erythrocytes of the peripheral circulation fail to incorporate vitamin B_{12} *in vivo* or when incubated with radioactive vitamin B_{12} *in vitro*.[2] These data may be interpreted to indicate that vitamin B_{12} is incorporated into the erythrocyte during the development of the cell in the bone marrow. In view of the essentially equal distribution of vitamin B_{12} between erythrocytes and plasma determined previously by microbiological methods, (Rosenthal and Brown, '54), the failure of red cells to incorporate the vitamin and the rapid loss of radioactivity from the reticulocytes as the cells age is difficult to explain at this time and must wait further study. However, it is conceivable that vitamin B_{12}, previously incorporated into the cell during formation, may diffuse out of the cell to the plasma but not the reverse.

The regular decrease of radioactivity in plasma following intramuscular or intravenous injection of radioactive vitamin B_{12} exhibits two decay curves that are exponential in character (fig. 2). The most rapidly disappearing curve exhibits a biological half life ($T\frac{1}{2}$) of 4.0 days. This value is not significantly altered by previous treatment such as hemorrhage anemia ($T\frac{1}{2} = 4.4$ days) or phenylhydrazine anemia ($T\frac{1}{2} = 3.6$ days). A second component with a slower rate of turnover becomes evident 12 days after injection. This component has a half life of 90 days for intravenous and 26 days for intramuscular injection. Since the radioactivity present in the plasma during the last 14 days of the experiment is quite low, with counting errors of the order of 10%, the difference between the rate of turnover for the intravenous or intramuscular route of administration of the slow component is of doubtful significance. On the other hand, the slow component for

[2] Unpublished results.

animals made anemic with phenylhydrazine (T ½ = 14.3 days) or hemorrhage (T ½ = 11.2 days) has a shorter half life than control animals. This may reflect an alteration of the slow component by the experimental treatment.

A component with a biological half life of 5 days, comparing favorably with that found in rabbit plasma, has been

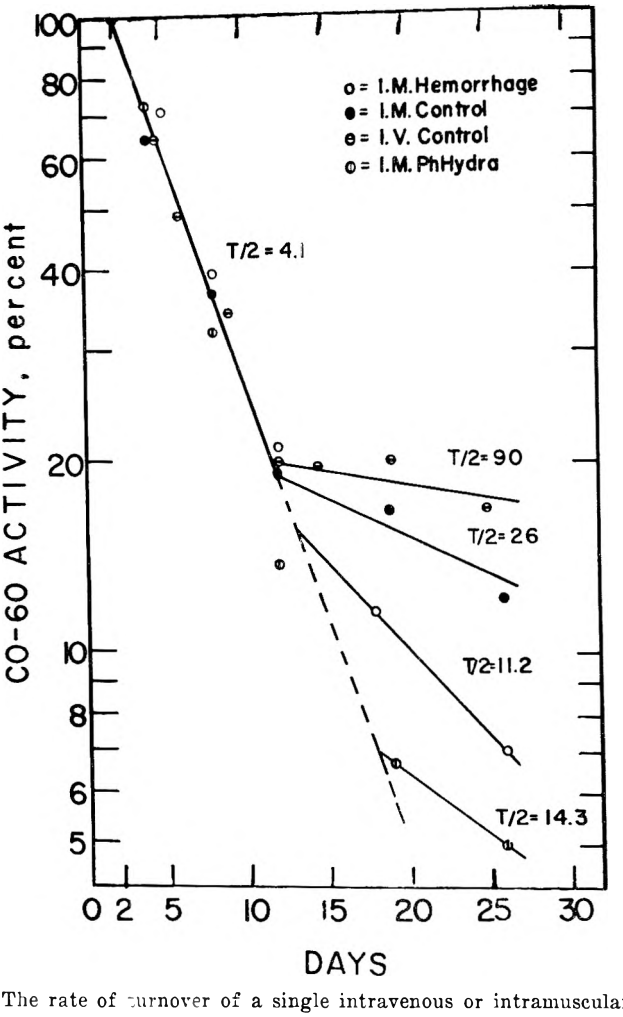

Fig. 2 The rate of turnover of a single intravenous or intramuscular injection of Co^{60} vitamin B_{12} in plasma of control (⊖, ●), hemorrhage anemia (○) and phenylhydrazine anemia (⊖) rabbits. The plasma radioactivity two days after dosage taken as 100%.

shown to be present in human subjects suffering from chronic myelogenous leukemia (Miller et al., '57). However, the slow component present in rabbit plasma, which exhibits a longer half life, has not been previously demonstrated in human subjects or other mammals, to our knowledge.

The animals from the previous experiments were sacrificed at the end of 26 days and the radioactivity of the organs was compared (table 2). The radioactivity present in the organs and tissues of the rabbit is not significantly altered by the various experimental procedures described in the report. However, in animals treated with phenylhydrazine, muscle accumulates significantly less vitamin B_{12} ($P < 0.01$) than the other groups of animals, but the significance of this observation is obscure.

The excretion of large amounts of vitamin B_{12} activity in the urine and feces of rabbits (Rosenthal and Cravitz, '58; Kulwich et al., '52) suggested that rabbit tissues may be saturated with the vitamin and excess vitamin B_{12} absorbed from the gut is rapidly excreted. In order to test this possibility, rabbits were injected intramuscularly with 500 μgm of non-radioactive cyanocobalamin daily for 4 days prior to the injection of the radioactive form. The radioactive material was given on the 5th day, 24 hours following the last non-radioactive vitamin injection. Other rabbits were starved for 10 days prior to the administration of radioactive material. Control animals were taken directly from stock and injected with Co^{60} vitamin B_{12} without any preliminary treatment. Urine and feces were collected for three days, the animals were sacrificed and the radioactivity of the tissues was determined.

The animals fortified with cyanocobalamin excreted 10% of the injected radioactivity in urine as compared with 4% for the controls (table 3). The starved animals also excreted a significantly greater percentage of the dose. In all cases, feces contained negligible quantities of radioactivity. Liver tissues from starved animals accumulated more of the injected dose than the control animals but the radioactivity of liver tissue from fortified animals was not altered. This is in ac-

cord with previous data (Rosenthal and Cravitz, '58). However, the kidneys of fortified animals accumulated 7 times more and those of starved animals 23 times more of the injected dose than the control animals. All of the other tissues studied were not significantly different from the controls.

TABLE 3

The effect of various treatments on tissue storage and urinary excretion of cobalt-60 vitamin B_{12} following intramuscular injection

TISSUE	CONTROL	FORTIFIED [1]	STARVED
Liver	10.71 ± 1.23 [2]	10.70 ± 0.59	40.19 ± 3.99 [3]
Kidney	14.25 ± 3.59	102.80 ± 31.80 [3]	318.30 ± 95.80 [3]
Muscle	2.76 ± 0.28	2.59 ± 0.32	2.29 ± 0.32
Heart	5.41 ± 0.61	9.40 ± 1.37	7.49 ± 1.51
Spleen	12.64 ± 1.92	14.00 ± 2.96	16.25 ± 1.33
Brain	2.45 ± 0.44	1.74 ± 0.35	2.49 ± 0.26
Urine	3.96 ± 0.27	10.05 ± 1.28 [3]	8.53 ± 1.61 [3]

[1] Animals fortified with 2 mg non-radioactive vitamin B_{12}.

[2] Mean values ± S.E. determined on 4 to 6 animals three days following injection. Values for tissues expressed in terms of the percent of the dosage present in 100 gm. Values for urine expressed as the percent of the dose excreted in three days. The animals were not collared.

[3] Values significantly different from controls at 1% level.

The finding that the kidneys from fortified animals accumulate more of the radioactivity is somewhat surprising and is difficult to explain. In similar experiments performed in human subjects (Meyer et al., '56), the accumulation of injected radioactive vitamin B_{12} in the kidney was not altered by injection of non-radioactive vitamin B_{12} except when non-radioactive vitamin B_{12} was given at about the same time as the radioactive vitamin. It would appear, therefore, that the metabolism of vitamin B_{12} by rabbit kidney is markedly different from that in human kidney.

Although the accumulation of vitamin B_{12} in kidneys from starved rabbits is associated with the excretion of the vitamin, the present findings that large doses of vitamin B_{12} also result in accumulation of the vitamin strongly suggest that the kid-

neys of rabbits play an important role, other than their function as excretory organs, in the metabolism of vitamin B_{12}.

In a previous communication (Rosenthal and Cravitz, '58), it was shown that starvation of rabbits for 13 days resulted in increased vitamin B_{12} activity in kidney, liver and heart tissue but the spleen vitamin concentration remained unaltered. These data, based on the assumption that intestinal vitamin B_{12} synthesis and absorption were minimal during starvation, were interpreted as indicating mobilization of the vitamin from muscle and other labile body stores. In order to test this assumption, rabbits were injected intramuscularly

TABLE 4

The effect of starvation on mobilization of cobalt-60 vitamin B_{12} in rabbit tissues

TISSUE	CONTROL A	STARVED	CONTROL B
Liver	16.48 ± 1.37 [1]	23.52 ± 2.17 [2]	13.52 ± 1.70
Kidney	14.20 ± 0.80	43.29 ± 7.26 [2]	15.58 ± 2.99
Muscle	2.89 ± 0.23	3.70 ± 0.48 [2]	2.79 ± 0.32
Heart	4.85 ± 0.73	6.91 ± 0.62	5.79 ± 0.83
Spleen	7.88 ± 0.69	5.39 ± 0.72	4.82 ± 1.36
Brain	4.28 ± 0.54	3.88 ± 0.56	—

[1] Mean values ± S.E. determined on 6 animals. All values expressed as percent of the dosage present in 100 gm of tissue. See text for details.

[2] Significantly different from controls at 1% level.

with Co^{60} vitamin B_{12} and allowed to equilibrate for 15 days. After 15 days, the animals were divided into three groups. One group (control A) was sacrificed for the determination of control Co^{60} vitamin B_{12} tissue values. A second group was starved for 10 days before sacrifice and the third group (control B) was sacrificed after 25 days, thus acting as a control for tissues that normally turn over vitamin B_{12} at a significant rate. As shown in table 4, starvation resulted in increased Co^{60} vitamin B_{12} concentration in liver and kidney tissue, thus indicating the mobilization of radioactivity from other tissue stores. The changes in the other tissues are of doubtful significance. Since the decrease in weight during 10 days of starvation is associated primarily with a loss of mus-

cle mass, fat stores and gastrointestinal tissues, we assume that these tissues furnish most of the labile, readily mobilizable quantities of vitamin B_{12}.

SUMMARY

In rabbits the rate of turnover of Co^{60} vitamin B_{12} during a 26-day period is negligible in liver, kidney, heart, skeletal muscle and brain, while in plasma and spleen the rate of vitamin turnover is rapid. The plasma disappearance curve of Co^{60} vitamin B_{12} exhibits two components with biological half lives of 4.4 days and approximately 50 days. Erythrocytes of normal rabbits fail to incorporate injected Co^{60} vitamin B_{12} but the young reticulocytes of animals made anemic with phenylhydrazine or hemorrhage incorporate the vitamin rapidly. The radioactivity of the reticulocytes is transient and leaves the cells as the reticulocytes mature. The uptake of the injected vitamin by other tissues is not altered in the anemic animals.

The accumulation of radioactivity in kidney tissue is markedly elevated above that of control animals when rabbits are starved for 10 days or fortified with injections of non-radioactive vitamin B_{12} prior to the injection of Co^{60} vitamin B_{12}. The incorporated Co^{60} vitamin B_{12} may be mobilized from the tissues by 10 days of starvation. This mobilization is variable, and differs for different tissues suggesting that vitamin B_{12} is intimately associated with the specific metabolism of individual tissues. The data also suggest a more fundamental role of the kidney in vitamin B_{12} metabolism in addition to the excretory function of the kidney.

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THE EFFECT OF AUTOCLAVING SOYBEAN PROTEIN AND THE ADDITION OF ETHYLENEDIAMINETETRACETIC ACID ON THE BIOLOGICAL AVAILABILITY OF DIETARY ZINC FOR TURKEY POULTS

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O'Dell and Savage ('57) demonstrated that dietary zinc is essential for chicks fed a ration containing isolated soybean protein.[1] However, no growth response to zinc was obtained if soybean protein was replaced by casein or by alpha protein. Roberson and Schaible ('58) were also able to produce an extreme deficiency of zinc in chicks by using a ration containing acid extracted soybean protein.[1] Morrison and Sarett ('58) observed that the zinc requirement of the chick was greater when a soybean protein [1] ration was used than when casein and gelatin were the sources of protein. Moeller and Scott ('58) found that the zinc requirement of chicks was less on an egg white diet than on a ration containing soybean protein. Pensack et al. ('58) observed that zinc was equally available for the chick from a variety of zinc salts and proteinates. A casein-gelatin basal ration was used in these studies. Norris et al. ('58) also observed that the zinc in soybean protein was not available for the chick.

Supplee et al. ('58) observed that zinc and potassium were needed for growth, prevention of perosis, and normal feathering in poults fed a soybean protein ration. Kratzer et al.

[1] Drackett C-1, The Drackett Products Company, Cincinnati, Ohio.

('58) confirmed the importance of zinc in rations for poults. Supplee et al. ('58) mentioned that, in a number of tests with steam sterilized, washed soybean protein replacing washed soybean protein in the ration, the incidence of abnormal hocks was reduced and feathering was improved.

In the present study we have attempted to determine the relationship of soybean protein and dietary zinc requirement of turkey poults, and to study factors which affect the availability of zinc in rations containing this protein.

EXPERIMENTAL

The basal ration contained the following: isolated soybean protein,[1] 33.0 gm; cellulose,[2] 5.0 gm; soybean oil, 3.5 gm; dicalcium phosphate ($CaHPO_4$), 3.0 gm; mineral mixture, 2.56 gm; calcium carbonate, 2.5 gm; vitamin mixture (Kratzer et al., '49) 2.0 gm; choline chloride (25%), 1.0 gm; DL-methionine, 0.45 gm; vitamin E concentrate (44 I.U./gm), 0.2 gm; vitamin D_3 concentrate (1,500 I.C.U./gm), 0.1 gm; inositol, 0.1 gm; vitamin A concentrate (20,000 I.U./gm), 0.05 gm; chlortetracycline, 0.01 gm; niacin, 2.0 mg; folic acid, 0.5 mg; biotin, 0.04 mg; vitamin B_{12}, 1 μg; and pearl corn starch to equal 100 gm. The mineral mixture supplied the following in grams per 100 gm of diet: NaCl, 1.0; $MnSO_4 \cdot H_2O$, 0.03; Na_2SiO_3, 0.110; $FeSO_4 \cdot 7H_2O$, 0.07; $CuSO_4 5H_2O$, 0.008; $Co(CH_3COO)_2 \cdot 4H_2O$, 0.002; KI, 0.001; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.025; $MgSO_4 \cdot 7H_2O$, 0.63; KCl, 0.3; K_2HPO_4, 0.381; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.001. Six samples of an identical basal ration contained an average of 25.5 p.p.m. of zinc as determined by the method of Rush and Yoe ('54).

Broad Breasted Bronze poults were fed the basal ration starting at hatching for about 4 days before being divided into groups of 10 poults each to be fed the experimental rations. The poults were housed in electrically heated galvanized batteries with wire floors and were provided feed and tap water ad libitum. They were weighed twice each

[1] See footnote 1, p. 313.

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

TABLE 1

Effect of treatment of soybean protein[1] (Drackett) and the addition of zinc upon the growth and perosis score of poults

PROTEIN SOURCE	ADDED ZINC	AVERAGE GAIN AND PEROSIS SCORE[2]			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
	p.p.m.	*gm*	*gm*	*gm*	*gm*
Soybean	0	167 (3.1)	150 (2.8)	144 (2.9)	
Soybean	57	348 (0.7)	319 (0.8)		
Soybean	60			305 (1.1)	
Soybean, autoclaved ½ hr.	0	253 (2.7)	207 (0.9)		
Soybean, autoclaved, 2 hr.	0			199 (3.2)	
Soybean, autoclaved, 2 hr.	60			275 (0.5)	
Soybean, heated ½ hr.	0	174 (3.2)	190 (3.3)		
Soybean, H_2O treated	0	187 (3.3)	157 (2.9)		
Soybean + arginine[3] + lysine[4]	0			149 (3.0)	
Soybean + arginine + lysine	60			303 (1.0)	
Soybean + arginine + lysine + methionine[5]	0				123 (2.6)
Soybean + arginine + lysine + methionine	50				303 (1.2)
Soybean, autoclaved 1 hr. + arginine + lysine	0			209 (3.4)	
Soybean, autoclaved 1 hr. + arginine + lysine	60			314 (0.8)	
Soybean, autoclaved 1 hr. + arginine + lysine + methionine	0				172 (3.3)
Soybean, autoclaved 1 hr. + arginine + lysine + methionine	50				280 (1.3)
Duration, days		21	19	19	19

[1] Drackett C-1, The Drackett Products Company, Cincinnati, Ohio.
[2] Perosis score within parentheses.
[3] L-Arginine, 0.3% of diet.
[4] L-Lysine, 0.5% of diet.
[5] DL-Methionine, 0.45% of diet.

week, and the experiments were continued for 19 to 22 days in the various trials. Perosis was scored according to the scale used previously (Kratzer et al., '58) in which values from 0 to 4 indicated normal to severely perotic poults.

In table 1 the results of 4 experiments show that the basal rations without added zinc gave very poor growth and the poults responded to the addition of approximately 60 p.p.m. of added zinc. When soybean protein was autoclaved at 15 pounds pressure per sq. in. for one-half hour before being mixed in the diet, growth of the poults was improved and perosis was reduced. On the other hand, if the soybean protein was heated in closed containers in an autoclave at 15 pounds pressure for one-half hour (dry-heated), or was wet with an equal weight of water and dried at 60°C, it was less effective in improving growth or preventing perosis. Diets unsupplemented with amino acids containing soybean protein autoclaved for two hours produced white bars in the feathers of the poults to which they were fed. This was presumably due to a reduction in available lysine as a result of autoclaving and was not noted in groups to which lysine was added. A response to autoclaving was still noted when arginine and lysine or arginine, lysine and extra methionine were added to the diets.

Since the autoclaving of soybean protein improved growth in a zinc-deficient diet, the zinc requirement of poults was determined with a ration containing the autoclaved protein and compared with the requirement as determined with a ration containing the unautoclaved protein. In figure 1 (experiment 5) the level of added zinc was varied from zero to 50 p.p.m. in rations containing either raw or autoclaved soybean protein. The requirement for zinc appeared to be definitely less with the use of the diet containing autoclaved soybean protein in comparison with the results obtained with the control, containing untreated protein suggesting that zinc was more available in the autoclaved protein.

Zinc forms complexes with many proteins and it was suspected that the zinc in the soybean protein was unavailable

due to a binding effect of the protein. The availability of the zinc in this protein was compared with that of a complex such as that formed with ethylenediaminetetracetic acid (EDTA). In experiment 6 (table 2), the zinc derivative of EDTA was formed and fed at a level equivalent to 19 p.p.m. of zinc with

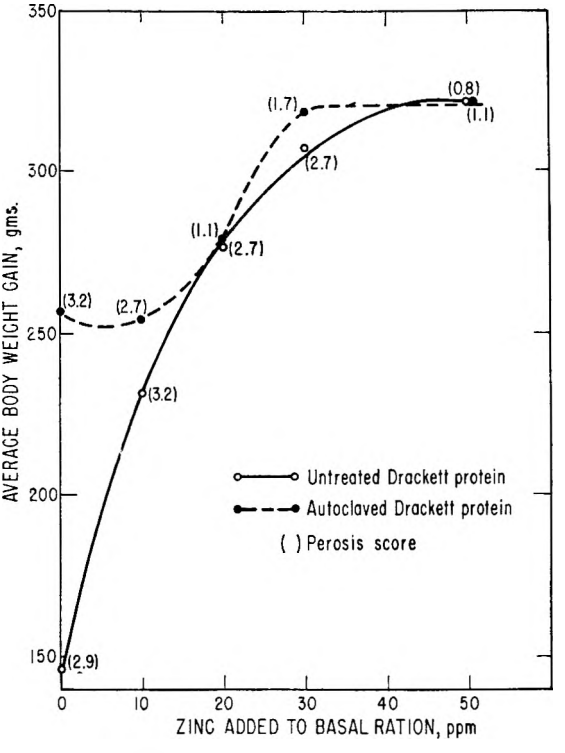

Fig. 1 Effect of autoclaving soybean protein (Drackett C–1) upon the requirement of zinc for the growth and prevention of perosis in poults.

free zinc and free EDTA used as controls. Supplements of zinc, the zinc complex and also the EDTA by itself improved growth. This was tested in further trials and it was confirmed that EDTA improved growth and at high levels reduced perosis when fed in a ration containing soybean protein. A reduction in the level of calcium in the ration (experiment 7) caused only a slight growth response as compared with

that obtained by EDTA. In experiment 9 the effects of various levels of zinc were compared with and without EDTA. Optimum growth and perosis prevention were obtained only at the highest level of zinc that was fed, whereas EDTA re-

TABLE 2

The effect of ethylenediaminetetracetic acid (EDTA) on the growth and incidence of perosis in poults fed rations containing isolated soybean protein [1] and various levels of zinc.

ADDED ZN	ADDED EDTA [2]	BODY WEIGHT GAIN AND PEROSIS SCORE [3]			
		Exp. 6	Exp. 7	Exp. 8	Exp. 9
p.p.m.	*p.p.m.*	*gm*	*gm*	*gm*	*gm*
0	0			170 (2.6)	162 (2.4)
11	0	218 (2.9)	184 (2.9)		
20					259 (2.9)
30	0	261 (2.4)	308 (3.1)		
40					272 (1.8)
60	0			315 (1.0)	
150	0				288 (0.8)
0	227			238 (3.0)	308 (0.9)
11	227	300 (1.8)	311 (3.0)		
11	454		320 (1.3)		
30	227 [4]	287 (0.9)	339 (1.2)		
20	227				295 (1.4)
40	227				294 (0.8)
60	227			327 (1.0)	
150	227				297 (1.2)
11	0		219 (2.9)[5]		
Duration, days		18	19	19	19

[1] Drackett C-1.
[2] Ethylenediaminetetracetic acid, disodium salt.
[3] Perosis score within parentheses.
[4] Fed as Zn complex equivalent to 19 p.p.m. Zn.
[5] $CaCO_3$ in diet reduced by 1%.

duced the requirement of zinc for optimum growth and perosis protection.

Poults which were fed a ration deficient in zinc developed an enlarged hock condition which has not been differentiated from perosis (Kratzer et al., '58). Poults from various experimental groups in two experiments were sacrificed and the legs placed upon an x-ray plate and exposed to x-rays. From

these plates it was possible to measure the length of the tarsometatarsus and width of the joint across the center of ossification as viewed from the posterior position. The ratios of the width to the length, shown in table 3, were significantly reduced by the addition of zinc, EDTA or the combination of both in experiment 9. The use of soybean proteins autoclaved for one hour (experiment 10) also reduced the above ratio by an amount which was significant at the 1% level.

DISCUSSION

Our results with poults confirm the previous reports that zinc is needed in chick rations which contain soybean protein

TABLE 3

Effect of zinc and ethylenediaminetetracetic acid (EDTA) supplements and autoclaving soybean protein[1] *on the ratio of width to length of tibiometatarsus of poults*

PROTEIN SOURCE	ZINC	EDTA	WIDTH/LENGTH	
			Exp. 9	Exp. 10
	p.p.m.	*p.p.m.*		
Soybean	0	0	0.36	0.37
Soybean	40	0		0.31
Soybean	150	0	0.28	
Soybean	0	227	0.29	
Soybean	150	227	0.29	
Autoclaved soybean, 1 hr.	0	0		0.34
Least significant difference				
P = 0.05			0.021	0.021
P = 0.01			0.029	0.028

[1] Drackett C-1.

as a source of protein. Our data also confirm the observation of Supplee et al. ('58) that abnormal hocks were rarely seen in poults fed a ration containing steam sterilized soybean protein while they were frequently observed in poults fed the ration containing untreated soybean protein and show that autoclaving the protein before feeding decreases the zinc requirement both for optimum growth and the prevention of

perosis in poults. Similar rates of growth were obtained when adequate zinc was added to both the treated and untreated diets containing soybean protein.

Ethylenediaminetetracetic acid is a strong chelating agent and has been widely used for this purpose. The results clearly show that EDTA is capable of improving the growth of poults fed a zinc-deficient diet containing soybean protein. The growth of poults fed EDTA and zinc was similar to that of those fed adequate zinc alone. The results thus indicate that EDTA is active in increasing the biological availability of zinc to the poult. This could be explained by assuming that EDTA is capable of removing zinc from combination with soybean protein. EDTA is a small enough molecule to be readily absorbed by the poult and thus the formation of the EDTA-zinc complex could render the zinc available for the bird. The possibility that EDTA reduces the level of available calcium ion which might interfere with zinc availability was shown not to be tenable since using a lower calcium level in the diet did not greatly improve the growth of poults.

The nature of the zinc-soybean protein complex is not known. There is evidence that zinc is bound to the imidazole group of histidine in human serum albumin (Gurd and Goodman, '52). Pensack et al. ('58) have shown that the zinc of zinc proteinates is available for the chick. It thus seems probable that the zinc is bound to a specific protein fraction of the soybean which is resistant to digestion in the bird, somewhat analogous to the unavailability of biotin in the presence of avidin.

The reduction in the width to length ratio of the tarsometatarsus by zinc or factors which increase zinc availability is similar to the observation by O'Dell et al. ('58) that a shortening and thickening of the long bones occurs in chicks deficient in zinc. Since the perosis scores were determined by subjective means, it was not possible to compare them statistically with the width-length measurements obtained from the x-ray plates.

SUMMARY

Autoclaving of the isolated soybean protein [3] used in a zinc-deficient ration improved the growth of poults. The addition of ethylenediaminetetracetic acid (EDTA) to a diet containing the soybean protein also reduced the requirement for zinc. The ratio of the width of the tarsometatarsus to its length was significantly reduced by the addition of either zinc or EDTA to a basal ration containing soybean protein.

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THE EFFECT OF STORAGE ON THE VITAMIN B_6 CONTENT OF A PACKAGED ARMY RATION, WITH A NOTE ON THE HUMAN REQUIREMENT FOR THE VITAMIN

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Army packaged rations consist of a variety of items, most of which are similar to civilian canned and packaged foods. Some rations, such as the 5-in-1 and C rations, are planned to provide all the nutrients required by physically active men. The preparation of such rations must take account of the chemical and nutritive changes produced by the high temperatures of processing. Changes known to occur are the "browning reaction," the destruction of vitamin C, thiamine, and also vitamin B_6 (Hassinen, Durbin and Bernhart, '54; Tomarelli, Spence and Bernhart, '55). Since army rations are often stored for considerable periods of time before issue, sometimes at rather high temperatures, the possibility of further nutrient losses must be considered. The changes in nutrient content of stored rations have been reviewed by Spector ('54) and Cannon ('54).

There is considerable evidence from animal feeding experiments that certain packaged rations may not contain optimal amounts of vitamin B_6 (Sporn, Ruegamer and Elvehjem, '48; Sporn and Elvehjem, '48; Register et al., '50; Tappan and Elvehjem, '53). The addition of the vitamin improves

such rations most markedly (Register et al., '50; Tappan and Elvehjem, '53). Due to the special requirements of each species, the further addition of folic acid and choline to the diet of monkeys (Sporn et al., '48) and casein to the diet of rats (Sporn and Elvehjem, '48) is required. The mortality of chicks fed C ration is reduced by the addition of pyridoxine, biotin and pantothenic acid (Scott et al., '54). These results are not directly applicable to man, since the human requirement for vitamin B_6 is not known and the need for this vitamin and for other nutrients varies with different species.

Kark et al. ('44) noted some symptoms and loss of physical fitness in military personnel subsisting for a long time on packaged rations. However, no such changes were observed in later field trials conducted by this laboratory (Johnson and Kark, '47; Ryer et al., '54; Welch et al., '57). It is possible that some of the original findings were caused by changes in the ration produced by storage.

The present work was designed to test the possibility that prolonged storage of a packaged ration could reduce the vitamin B_6 content enough to produce evidence of deficiency. Volunteer human subjects consumed C rations that had been stored for 20 months under two conditions: at 34° and 100°F. Xanthurenic acid excretion after a tryptophan load (Greenberg et al., '49) was determined as a measure of vitamin B_6 sufficiency. This experiment was part of a larger study of acceptability, digestibility, metabolizable energy and composition of the C ration, as affected by storage of the ration (Plough et al., '58).

EXPERIMENTAL

The test subjects were 9 male conscientious objectors, ages 20 to 28, height 168 to 188 cm, and weight, 65 to 83 kg, who were housed in a metabolic ward. They consumed the rations completely, and had no other source of food. Body weights were maintained relatively constant by an exercise program designed to utilize the 500 to 600 Cal. provided by the ration above the usual requirement of life on the metabolic ward.

The C ration employed was procured in 1954. Although individual items came from many factories, each represented a single batch. The items were assembled simultaneously with each carton containing 6 rations, and subsequently stored for 20 months at the Quartermaster Food and Container Institute. Alternate cartons were stored at 34°F. (C–1 ration) and at 100°F. (C–2 ration). A control diet designated the A ration was prepared from locally procured, fresh foods to match the macronutrient intake of the C rations.

Each subject was fed the A, the C–1 and C–2 rations. The rations were fed for 24 days each, with an 11-day rest period between each feeding cycle. The 6 possible sequences of feeding of the three rations made up two 3×3 Latin squares. A third square was a duplicate of the first. The 9 subjects were assigned at random to a place in each square.

Tryptophan load tests were performed at the beginning, and after 18 and 24 days of each diet. Twenty-four-hour urine collections were made before and after the ingestion of 10 gm of DL-tryptophan (Greenberg et al., '49). Xanthurenic acid was determined by the method of Rosen, Lowry and Sprince ('51) as modified by Wachstein and Gudaitis ('52) but with one change: 5 ml each of urine and buffer were used instead of 10 ml. Composites of each menu of each ration were subjected to proximate analysis by AOAC procedures ('55). The vitamin B_6 content was assayed microbiologically by the method of Atkin et al. ('43) using *Saccharomyces carlsbergensis* no. 4228.

RESULTS

The macronutrient content of the three rations is recorded in table 1. There was little difference between the two C rations, but the A ration contained less of all three macronutrients than did the C ration. This came about because the A ration was made up to match the procurement specifications of the C ration; the C ration actually contained greater amounts than required by the specifications.

The A ration contained 4.28 mg of vitamin B_6. The average content of the C–1 ration was 2.76 mg, and of the C–2 ration

1.93 mg (table 2). Although the ranges of values of the vitamin B_6 content of the two rations overlap, each menu of the C-2 ration contained considerably less of the vitamin than did the corresponding menu of the C–1 ration. The mean of the differences between menus is statistically different from zero.

TABLE 1

Macronutrient composition of rations as determined by analysis

RATION	PROTEIN	FAT	CARBOHYDRATE	METABOLIZABLE ENERGY [1]
	gm	*gm*	*gm*	*Cal.*
A	149	134	476	3706
C-1 [2]	164	142	487	3882
C-2 [2]	165	141	499	3925

[1] Metabolizable energy calculated from macronutrient composition by the use of Atwater's 4–9–4 factors.

[2] Mean of six menus.

TABLE 2

Vitamin B_6 content of the rations

C-1 RATION		C-2 RATION	
Menu	Vitamin B_6	Menu	Vitamin B_6
	mg		*mg*
1	2.11	1	1.57
2	2.93	2	1.58
3	2.94	3	2.02
4	2.25	4	1.99
5	3.33	5	2.17
6	2.97	6	2.27
Mean	2.76	Mean	1.93
A ration		4.28 mg vitamin B_6	

The basal excretion of xanthurenic acid averaged 6 to 8 mg per day. The net excretions of xanthurenic acid by the test subjects (amount excreted the day of tryptophan administration minus the amount excreted the day before) are listed in table 3. By analysis of variance of the data, ration was a significant source of variation (probability of non-significance less than 0.05), while time and men were not. The difference in mean net excretion of xanthurenic acid between the C–1

TABLE 3

Net excretion of xanthurenic acid in response to tryptophan ingestion (milligrams per 24 hours)

SUBJECT	A RATION				C-1 RATION				C-2 RATION			
	Control	18 Days	24 Days	Cycle [1]	Control	18 Days	24 Days	Cycle [1]	Control	18 Days	24 Days	Cycle [1]
A	6	— 3	7	1	— 5	13	12	2	12	23	63	3
B	38	10	19	2	6	9	19	3	5	68	55	1
C	3	5	— 4	3	4	4	34	1	15	16	28	2
D	12	— 4	16	1	12	20	— 2	3	4	22	17	2
E	9	4	6	2	6	4	10	3	— 1	15	63	1
F	11	1	31	3	13	0	3	2	10	— 3	— 3	1
G	13	21	6	1	35	4	3	2	19	36	24	3
H	6	—	10	3	— 1	25	13	1	15	14	18	2
I	15	—	11	2	3	21	12	1	12	35	22	3
Mean	12.6	5.8	11.3		8.1	11.1	11.6		10.1	25.1	31.9	

[1] Feeding cycle in which subject received the ration.

and C–2 rations at 24 days was also significant. The high control values of subject B in the A ration period probably represents a carry-over of the effect of the C-2 ration which he had consumed up to 11 days before the control test for the A ration. The high value of subject G during the control test of the C–1 ration period is unexplained. No other evidence of vitamin B_6 deficiency was observed.

DISCUSSION

The data presented here demonstrate that appreciable losses of vitamin B_6 may occur in packaged rations undergoing prolonged storage at high temperature. The average vitamin B_6 content of the heat-stored C–2 ration, 1.93 mg, was probably comparable to that of the C rations used by others in animal experiments. If it is assumed that the latter rations were of the same weight (1718 gm per ration) and had the same moisture content (52%) as did those used in the present study. It may be calculated that the ration of Register et al. ('50) contained 2.02 mg, and that of Scott et al. ('54), 1.45 mg by microbiological assay and 1.74 mg by chick assay. These vitamin B_6 concentrations were sufficiently low to produce evidence of severe deficiency in rats and in chicks, respectively.

The present data also give some evidence of the level of the human requirement of vitamin B_6. After consuming 4.28 mg (A ration) or 2.76 mg (C–1 ration) of vitamin B_6 for 24 days, none of the 9 test subjects showed any increase in xanthurenic acid excretion after tryptophan ingestion; but after consuming 1.93 mg (C–2 ration) for 24 days, 7 out of 9 subjects did show increases in xanthurenic acid excretion. The upper range of net excretion of xanthurenic acid by the normal subjects studied by Wachstein and Gudaitis ('52) was 74 mg. None of the test subjects in the present report exceeded this level, although three men approached it. If these small evidences of disturbance in tryptophan metabolism are accepted as evidence of vitamin B_6 deficiency, then an average intake of 1.93 mg per day of vitamin B_6 was below the daily require-

ment of the test subjects. On the other hand 2.76 mg was an adequate daily intake. This human daily requirement is within the wide range of 0.5 to 5.0 mg estimated by Vilter et al. ('53) from work with deoxypyridoxine, but is higher than the estimate of 1 to 2 mg by the National Research Council ('58). It must be stated, however, that there is no evidence that increased excretion of xanthurenic acid after tryptophan load *per se* is detrimental to the organism. It should also be noted that the test subjects were receiving a high-protein diet and that the fat of the ration (32% of the calories) was largely of animal origin and, therefore, relatively saturated. Both of these factors may conceivably increase the requirements for vitamin B_6, since the vitamin is known to be involved in transamination and in the interconversion of saturated and unsaturated fat.

SUMMARY AND CONCLUSIONS

An army C ration that had been stored (after assembly) for 20 months at 34°F. contained an average of 2.76 mg of vitamin B_6 per ration by microbiological assay. Other rations from the same assembly that had been stored for the same length of time at 100°F. contained an average of 1.93 mg of vitamin B_6.

These two C rations, together with a ration of similar macronutrient composition prepared from fresh food, were fed for 24 days each to 9 young male test subjects. At the end of the period of consumption of the C rations, the average net excretion of xanthurenic acid, after ingestion of 10 gm of DL-tryptophan, was 11.6 mg in 24 hours in the case of the ration stored at 34°F., and 31.9 mg for the ration stored at 100°F. as compared to 11.3 mg for the control ration.

Under the conditions of the experiment the minimum daily requirements of the test subjects for vitamin B_6 was between 1.93 and 2.76 mg.

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