RED BLOOD CELL NIACIN AND PLASMA RIBOFLAVIN LEVELS IN A GROUP OF NORMAL CHILDREN [1]

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FOUR FIGURES

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Blood levels of some of the vitamins have been determined as part of a long-range study of normal children. The data presented here are the results of determinations of the red blood cell niacin and the total plasma riboflavin. Both series of determinations were made over a period of approximately 5 years, but have been discontinued.

MATERIAL AND METHODS

For the niacin determinations, a group of 86 Child Research Council children have been studied, of whom 37 were boys and 49 were girls. For the plasma riboflavin determinations, a group of 92 Child Research Council children were studied, of whom 39 were boys and 53 were girls. In both studies, ages ranged from two days to 16 years. Four or more determinations were made on each child. In addition to these, 185 niacin and 120 riboflavin determinations were done on children from the Denver Orphans Home, The Colorado State Home, the Well Baby Clinic, the Florence Crittenton Home and the Children's ward at Colorado General Hospital.[2]

[1] This study was supported in part by the Nutrition Foundation.

[2] Acknowledgment is extended to the staffs of these institutions for their helpful cooperation.

Determinations were made at two days, one, two and three months of age, at three months' intervals thereafter until three years of age, and at 6 months' intervals from three years on. Blood was taken at varying hours of the day, so that no fasting levels were obtained. Finger prick blood was used in the majority of cases, venipuncture blood in the other cases. No difference in vitamin levels between peripheral and venous blood has been observed. The total number of determinations was 769 for niacin and 848 for riboflavin. The number at each stated age level varied from 16 to 26.

The dietary histories referred to were taken on the Child Research Council children of 10 years or under, and, in a few cases, on older children. These were obtained through home visits with the mother in order to determine the amount and kind of food eaten during a stated time interval. A record of 24-hour intakes was also obtained over a period of 4 days between home interviews. The data used here are the average daily intakes calculated on the basis of both the interval histories and 24-hour intakes.

The hematological studies referred to were done on the same blood samples as used for the vitamin studies. The peripheral or venipuncture blood is used for determinations of sedimentation rate, total red cell count, hemoglobin, cell volume, reticulocytes, total white cell count and differential count.

Niacin method. The method of Levitas et al. ('47) was used. The volumes of reagents were modified and the determinations carried out according to the following procedure:[3] All reagents are made with glass-redistilled water. Into 1 ml of saline, 0.05 ml of whole blood is pipetted, 3 ml of 15% trichloracetic acid added and the mixture allowed to stand for one-half hour before centrifuging. The filtrate is collected and will remain stable for several days in the refrigerator. The determinations are run in duplicate. For a single determination, 0.2

[3] The modification of this method was worked out by Betty Szymanski working in Dr. Bernard B. Longwell's laboratory. Mrs. Szymanski also carried out the earlier determinations reported in this study.

ml of filtrate is added to each of three 2-ml glass-stoppered volumetric test tubes. To tube 1 (sample, U) and tube 3 (blank, B) 0.2 ml of water is added; to tube 2 (standard, S) is added 0.2 ml of standard containing approximately 9.0 μg/ml of N-methyl nicotinamide which has been diluted 1 to 25 shortly before use. Then 0.1 ml of acetone is added to tubes 1 and 2 and the tubes are shaken immediately. To each of the tubes 0.14 ml of 6 N NaOH is added; the tubes are shaken and allowed to stand for 5 minutes. Then 0.2 ml of 6 N HCl is added to each tube, mixed well, and the tubes are placed in a boiling water bath for 4 minutes, removed and allowed to cool. One milliliter of 20% KH_2PO_4 is added to each tube and the volume made up to 2 ml. The contents of the tubes are mixed, transferred to fluorometer tubes, and read. Calculations are in terms of diphosphopyridine nucleotide since red blood cell niacin is present as coenzymes I and II (DPN and TPN) in the proportion of about 8:1.

$$\frac{U - B}{S - U} \times \frac{\mu g \text{ NMN} +}{\text{ml cells}} \times 2.53 = \mu g \text{ DPN/ml cells}$$

2.53 is the factor which corrects for the difference in the fluorescence between NMN + and DPN.

Riboflavin method. The method of Burch, Bessey and Lowry ('48) was used in order to determine total plasma riboflavin. The quantities of reagents were modified and the procedure used was as follows:[4] Five-hundredths milliliter of plasma is precipitated with 1.3% trichloracetic acid at room temperature and allowed to stand for 10 minutes. It is then centrifuged, transferred to a clean test tube and hydrolyzed in the dark at 37° for 20 hours. One milliliter is pipetted into a fluorometer tube and 0.5 ml of 1.2 M K_2HPO_4 added. After thorough mixing by bubbling air through the mixture, the initial reading, U, is taken in the fluorometer; 0.05 ml of riboflavin standard, containing 0.100 μg/ml, is added and the second reading, S, is made. One drop of freshly made 10% sodium hydrosulfite in 5% sodium bicarbonate is added and

[4] See footnote 3, page 158.

the reduced reading, B, is made. The calculation is then made as follows: [5]

$$\frac{U - B}{S - U} \times \frac{\mu g \text{ standard}}{\text{ml sample}} \times 100 = \mu g \text{ riboflavin/100 ml of plasma}$$

RESULTS

The results of the niacin and riboflavin determinations are presented in figures 1 and 2. Niacin, expressed as micrograms of diphosphopyridine nucleotide/ml of red cells, and riboflavin, expressed as micrograms/100 ml of plasma, have been plotted against age. All of the determinations are shown in the scatter graphs, as well as the smoothed medians. It will be observed that the initial high level of riboflavin and initial rise in niacin are both followed by a decline, after which the level remains within a fairly constant range. It will also be observed in both graphs that there is a wide range of normal values, with a somewhat skewed distribution in the case of riboflavin. There is no significant difference between the boys and girls at any age. The range of values obtained from children outside the Child Research Council is not different from the range of the children in the Council. Analysis of the graphs of the 86 to 92 individuals on whom 4 or more niacin or riboflavin determinations were made shows that, in approximately 75% to 80% of the cases, the individuals covered a range of three or 4 quartiles. About 20 to 25% of the individuals had niacin or riboflavin levels which showed a possible trend as indicated by a range limited to one or two quartiles. Figures 3 and 4 give

[5] A correction was made, in the calculation, to account for the dilution resulting from the addition of internal standard. S–U was multiplied by a factor of 1.5 ml + volume of standard/1.5 ml. It is possible to make this correction somewhat differently, with the result that the final values of plasma riboflavin would be reduced from 1 to 3%. No correction was made in reading B for dilution, since this reading corresponds very nearly to water and is unaffected by dilution. No reagent blank values were subtracted from the final tabulated values. Blank values were determined which varied from 0.0 to 1.0. It was felt, however, that the inherent variation in readings on our modified microphotofluorometer is such that these blank values are not precise enough, and, especially in low ranges of riboflavin, would introduce a greater error than they would remove, with possible misleading results.

examples of the type of individual considered to show some trend as compared with the majority of cases where no trend was apparent.

Using 4-fold tables for approximately 100 niacin determinations on children ranging from one to 7 years of age, there was no correlation between the direction of deviation from

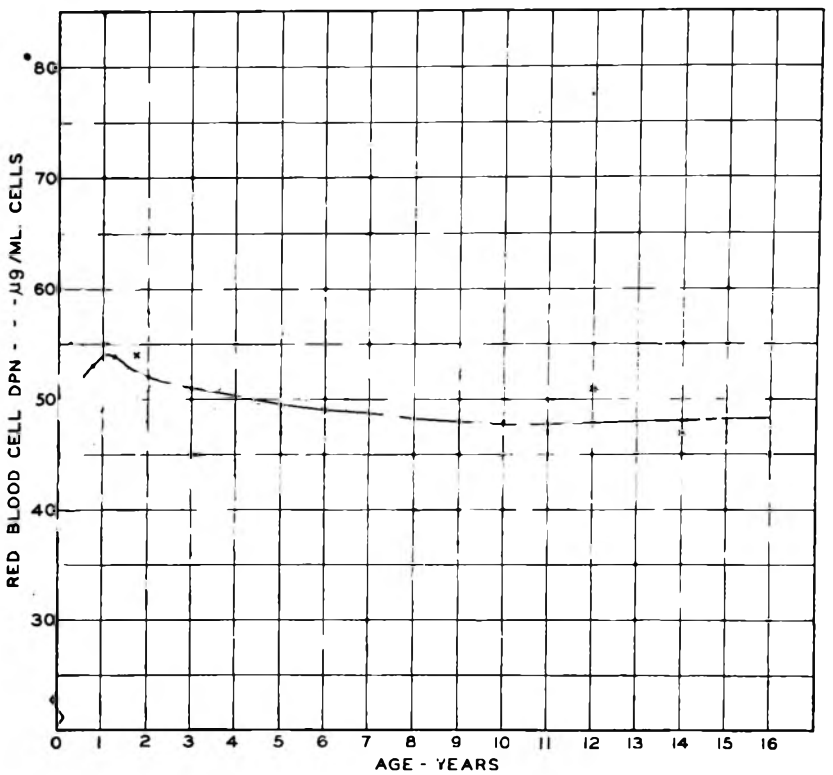

Fig. 1 Distribution of values of red blood cell niacin by age, showing smoothed median. The observed median at each age is indicated by x.

the median niacin intake per kilogram of body weight and the direction of deviation from the median red blood cell level. This was true when a supplementary vitamin concentrate was included in the intake and when it was excluded. There was also no correlation between absolute intake, inclusive or exclusive of concentrate, and the blood levels of niacin.

Using 4-fold tables with approximately 300 niacin determinations on children ranging from birth to 16 years of age, there was no correlation between the direction of deviation from the median blood cell levels and that from the median levels of hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration or reticulocytes.

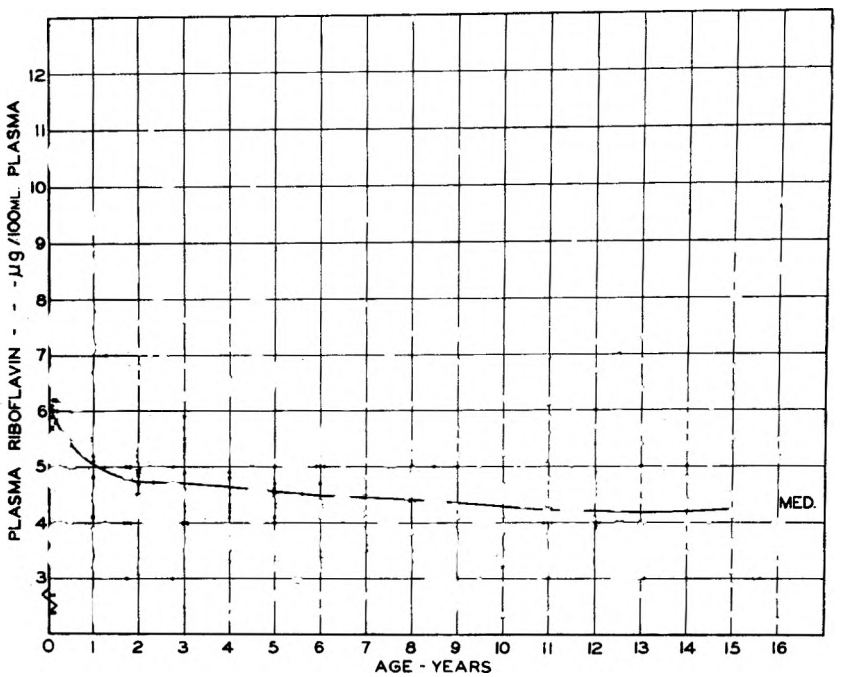

Fig. 2 Distribution of values of plasma riboflavin by age, showing smoothed median. The observed median at each age is indicated by x.

In the case of riboflavin, in order to compare intake with blood plasma level, 350 determinations were used which had been obtained from children ranging from one month to 10 years of age. The correlation coefficient for dietary intake per kilogram of body weight and plasma level was + 0.261. Comparing the direction of deviation from the median blood level with the direction of deviation from the median total dietary intake, total dietary intake plus supplementary vitamin concentrate, and the dietary intake per kilogram of body weight,

no significant correlation was found. In comparing the intake of those individuals receiving concentrate with their plasma level, there was no indication that under the conditions of this study a level of intake could be reached, above which it would, in general, be reflected by high plasma level. The range of blood levels for those receiving concentrate was no different from the range for those receiving no concentrate.

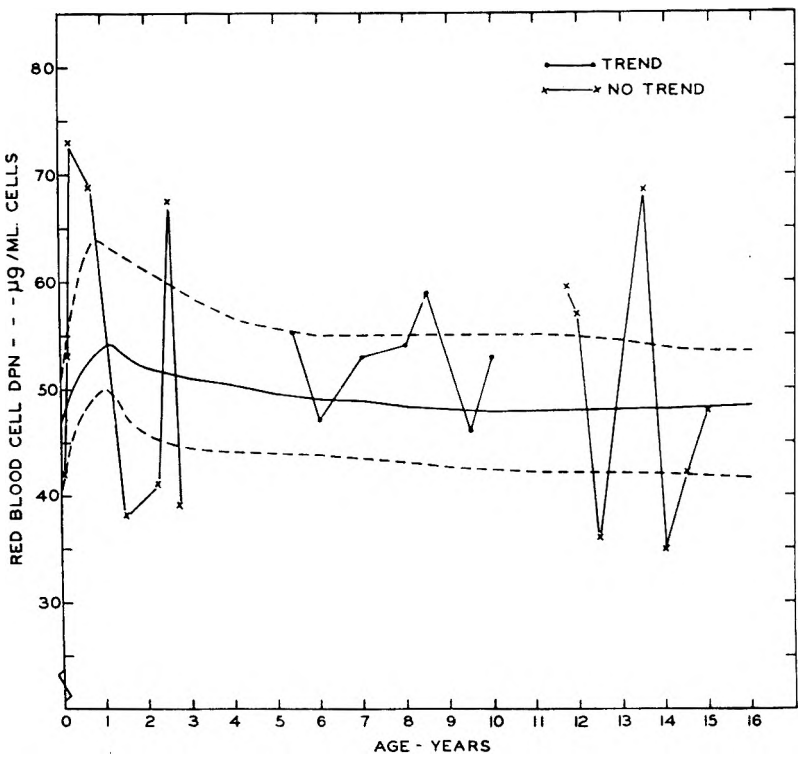

Fig. 3 Red blood cell niacin determinations in three children, plotted against the median (——) and the 25th and 75th percentiles (– – –).

DISCUSSION

The vitamin determinations were originally undertaken in conjunction with the dietary histories in an effort to determine whether or not, under dietary conditions which are considered "normal" and with extended study of each individual,

the blood levels would in some measure reflect the nutritive status of the individual with respect to the particular vitamin. If no indication of a relationship between intake and blood level exists under these conditions, is the blood level a reflection of a multitude of factors which relate primarily to the internal demands of the individual rather than the external supply? If the blood levels are maintained at a fairly stable level, is there any indication that, in certain individuals, ac-

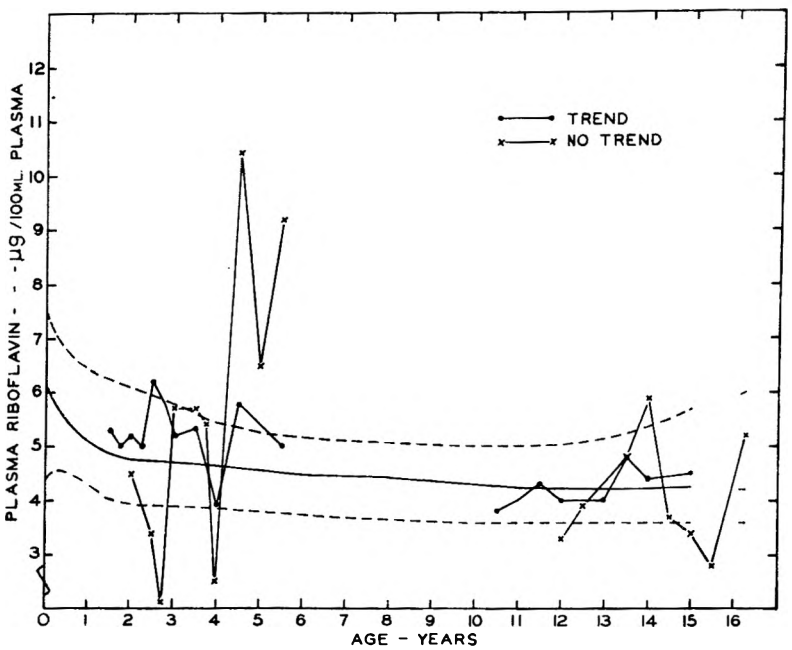

Fig. 4 Plasma riboflavin determinations on 4 children plotted against the median (——) and the 25th and 75th percentiles (– – –).

cepted dietary requirements are insufficient to maintain this level, or that different individuals have different normal levels? Is there a minimum above which fluctuations reflect intake? Does blood level change, for example, during periods of most rapid growth or some critical stage in the individual's development?

The results of the investigation of niacin seem to represent simply the range of variation for children receiving adequate

diets. Except for the first two years of life, the range remains quite constant. On an individual basis, for the most part, the separate determinations fall at random within this range, indicating a wide, unoriented fluctuation. Comparing these results with those from other laboratories, in general, these levels are somewhat lower. Levitas et al. ('47) studied 16 normal subjects from 20 to 30 years of age. The range of values was 62 to 89 μg of DPN/ml of cells. Klein et al. ('42), using a colorimetric method to determine nicotinic acid, found an average level corresponding to approximately 70 μg of DPN/ml of cells. Hoagland et al. ('42), using a microbiological method to measure the V factor, studied 60 normal subjects and found an average of approximately 50 to 70 μg/ml of cells. Kohn et al. ('39), measuring the V factor in 53 subjects, found 50 to 100 μg/ml of cells. Melnick et al. ('40), measuring nicotinic acid in whole blood, found a range of 5.4 to 8.3 μg/ml in men and 5.2 to 7.4 μg/ml in women, which, expressed as DPN, would give a total range for whole blood of approximately 26 to 42 μg/ml. The median of the determinations made in this laboratory on children between ages 4 and 16 years is somewhat less than 50 μg/ml of cells, with no significant difference between boys and girls.

The lack of correlation between niacin intake and blood cell level does not seem surprising since not only is the niacin intake presumably adequate, but no calculation has been made of tryptophan as a source of niacin. This subject has been reviewed by Krehl ('49) and it has been found that tryptophan probably accounts for a very high percentage of available niacin. Axelrod et al. ('40), Klein et al. ('42), Kohn et al. ('39a) and Kohn and Klein ('39b) have studied both niacin-depleted individuals and those who have received very large supplements of nicotinic acid over prolonged periods. Depletion produced no significant change in red cell coenzyme I. The results of giving very large supplements indicated that an increase of 10 to several hundred times normal intake is essential to produce significant changes from normal. It was also found that the higher the original level the less marked

the increase, indicating that probably there are other limiting factors beyond available niacin which determine the coenzyme level in the red cells. Furthermore, it has been suggested that while the blood coenzyme I remains relatively constant during deficiency, other tissues, including muscle and liver, reflect depletion (Axelrod et al., '40; Kohn et al., '39a). It would seem then that other tissues are in a more labile condition with respect to niacin, or that red cells are able to maintain at least a minimum level essential for normal metabolic processes at the expense of other tissues, long after serious depletion has occurred in other parts of the body. For this reason, it is probably not surprising that, even over long periods of time, there is no apparent relationship between hematological findings and niacin levels.

In the case of riboflavin, the wide scatter of levels found in the great majority of cases might indicate that several factors are affecting plasma riboflavin levels. Since total plasma riboflavin was determined, it is essential to consider the combined flavine adenine dinucleotide (FAD), which represents about 75% of the total, as well as a small percentage of flavine mononucleotide (FMN) and the free riboflavin which account for the remaining 25%. It appears from the work of Burch et al. ('48) that the combined FAD remains at a more stable level than the free riboflavin. It is probable that FAD reflects the more constant internal metabolic demands and therefore changes in the level of FAD may be indicative of the nutritional status of the body as a whole. On the other hand, free riboflavin, as the more labile portion, may reflect the immediate past intake.

The graph indicates that, at approximately 3.5 μg/100 ml, there is what might be regarded as a normal basal level, which may be similar to a fasting level. Similar values have been reported by Burch et al. ('48) and by Suvarnakich et al. ('52). Since there was no correlation between the level of riboflavin and prolonged relatively high intake over periods between blood sampling, it might be assumed that within a wide range of adequate intake, the high values are temporary and simply

indicate the amounts of riboflavin consumed before sampling. Klein and Kohn ('40) have shown that after ingestion of excessively large doses of riboflavin, the plasma levels return to normal within somewhat more than 24 hours. Therefore, under the conditions of the Child Research Council studies, variations in plasma levels cannot be interpreted as reflecting differences in nutritional status, utilization, or individual requirement, and are probably due largely to changes in the free plasma riboflavin.

Finally, the role of both niacin and riboflavin in the internal metabolic activities of the body have been considered. Both vitamins become parts of enzyme systems involved primarily in energy-supplying reactions in contrast, for example, with enzymes involved with bone growth. It would seem, therefore, that neither niacin nor riboflavin, as determined in this study, would be expected to change markedly or consistently in relation to growth. For this reason and those discussed above, we have discontinued these serial determinations in our longitudinal studies.

The data are presented here in the hope that the resulting picture of the ranges during health may be useful to others.

SUMMARY

Normal levels of red blood cell niacin, in a group of 86 children studied over a period of approximately 5 years, are presented. The results of these determinations are correlated with niacin intake and hematological studies. No positive correlation is found.

Normal levels of plasma riboflavin, in a group of 92 children studied over a period of approximately 5 years, are presented. The results of these studies are correlated with riboflavin intake. No positive correlation is found.

The results of both series of determinations are discussed in relation to theoretical considerations and in the light of other experimental findings.

LITERATURE CITED

AXELROD, A. E., E. S. GORDON AND C. A. ELVEHJEM 1940 The relationship of the dietary intake of nicotinic acid to the coenzyme I content of blood. Am. J. Med. Sci., *199:* 697.

BURCH, H. B., O. A. BESSEY AND O. H. LOWRY 1948 Fluorometric measurements of riboflavin and its natural derivatives in small quantities of blood serum and cells. J. Biol. Chem., *175:* 457.

HOAGLAND, C. L., AND S. M. WARD 1942 The quantitative determination of factor V by measurement of nitrate produced by hemophilus influenzae. Ibid., *146:* 115.

KLEIN, J. R., AND H. I. KOHN 1940 The synthesis of flavin-adenine-dinucleotide from riboflavin by human blood cells in vitro and in vivo. Ibid., *136:* 177.

KLEIN, J. R., W. A. PERLZWEIG AND P. HANDLER 1942 Determination of nicotinic acid in blood cells and plasma. Ibid., *145:* 27.

KOHN, H. I., F. BERNHEIM AND A. V. FELSOVANYI 1939a The blood V-factor (coenzyme) level in normal and pathological subjects. J. Clin. Invest., *18:* 585.

KOHN, H. I., AND J. R. KLEIN 1939b The synthesis of cozymase and of factor V from nicotinic acid by the human erythrocyte in vitro and in vivo. J. Biol. Chem., *130:* 1.

KREHL, W. A. 1949 Vitamins and Hormones, VII, III: Niacin in Amino Acid Metabolism.

LEVITAS, N., J. ROBINSON, F. ROSEN, J. W. HUFF AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N-methylnicotinamide and acetone. J. Biol. Chem., *167:* 169.

MELNICK, D., W. D. ROBINSON AND H. FIELD, JR. 1940 Factors affecting the concentration and distribution of nicotinic acid in the blood. Ibid., *136:* 157.

SUVARNAKICH, K., G. V. MANN AND F. J. STARE 1952 Riboflavin in human serum. J. Nutrition, *47:* 105.

SOME OBSERVATIONS ON VITAMIN METABOLISM IN GERM-FREE RATS [1]

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THREE FIGURES

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Under certain conditions of nutritional stress the white rat shows a dietary requirement for several vitamins which are apparently not needed under normal conditions. Deficiency syndromes in rats fed sulfa-drugs may be partially prevented or alleviated by feeding biotin, folic acid, p-aminobenzoic acid, vitamin K and ascorbic acid, as reviewed by Williams et al. ('50), whereas rats normally do not require dietary sources of these vitamins. These effects have often been attributed to interference with "intestinal synthesis." While it is established that certain bacteria can synthesize vitamins (Peterson and Peterson, '45), there is no unequivocal proof that non-ruminants utilize vitamins synthesized in the intestine. Experiments employing various drugs or surgical procedures actually add one or more variables to the experiment.

Germ-free animals provide an evaluation of the role of the intestinal microflora in nutrition. The success of Cohendy ('12) in rearing germ-free chicks opened the possibility for doing direct experiments in animals without microbial complication. Although the nutrition of the germ-free rat has

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR131-067.

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not been studied systematically, milk formulae have been used to rear these animals to weaning by Reyniers et al. ('46) and Gustafsson ('46). Results with semi-synthetic diets used in experiments with weaned germ-free rats by du Vigneaud et al. ('51), Orland et al. ('54) and Luckey et al. ('54) indicate that the general pattern of nutritional requirements of the germ-free rat is similar to that of the conventional rat.

The experiments reported herein were designed to obtain maximum information about the vitamin requirements of germ-free rats using a limited number of animals. For this purpose the following hypothesis was formed: germ-free rats may not require dietary sources of biotin, folic acid, citrovorum factor, vitamin B_{12}, vitamin K or inositol since uncomplicated deficiencies of these vitamins cannot be obtained in conventional rats. These vitamins were therefore omitted from the diet. Ascorbic acid, niacin and p-aminobenzoic acid were purposely fed to give optimum conditions for folic acid biosynthesis. When the pilot experiment indicated that folic acid could be synthesized in the presence of biotin, a vitamin balance study was run with biotin in the diet to obtain further evidence of tissue synthesis of folic acid.

METHODS

Germ-free albino rats were maintained in an environment completely free from all other forms of demonstrable living organisms as ascertained by standard bacteriological cultural and visual methods. Orland et al. ('54) report the methods in detail. Germ-free rats used in the pilot experiment were reared by hand feeding after Caesarian delivery. This experiment started November 14, 1950 when the rats were 46 days old. Pertinent data recorded herein were used to plan the balance study. The balance study was run with 6th generation germ-free rats born and suckled naturally. These rats were born November 15, 1952, fed the experimental diet at 25 days of age and maintained on the balance study from the 38th to the 188th day of life. The conventional rats were albino rats born and maintained in a conventional stock colony. All

rats were maintained on 1 cm wire screen and fed steam-sterilized food and water at libitum.

The composition of the most deficient diet (L-283) used is given in parts by weight: cornstarch, 590; casein [3], 250; corn oil, 70; salts L-II (Luckey, Pleasants and Reyniers, '55), 60; roughage,[4] 20; vitamin A esters, 8000 I.U.; ascorbic acid, 20; vitamin D_3, 1000 I.U.; α-tocopherol, 0.5; thiamine HCl, 0.05; riboflavin, 0.03; pyridoxine HCl, 0.02; pyridoxamine 2HCl, 0.004; Ca pantothenate, 0.3; nicotinamide, 0.05; nicotinic acid, 0.05; choline Cl, 2.0; and p-aminobenzoic acid, 0.05. Diet L-295 was prepared as above with the addition of biotin. Microbiological analyses on 7 samples of autoclaved diet L-295 taken from the germ-free cage at different periods during the balance study gave the following average concentrations: riboflavin, 0.035; Ca pantothenate, 0.24; nicotinamide, 0.11; inositol, 0.21; biotin, 0.00082; folic acid, 0.000011; citrovorum factor, 0.000014; and vitamin B_{12}, 0.0000034 gm/kg. The composition of the complete diet, L-109, was the same as diet L-283, with added biotin, 1 mg/kg; inositol, 1 gm/kg; folic acid, 10 mg/kg; vitamin K, 100 mg of menadione/kg; 2% yeast powder and 3% liver powder.

The amount of food eaten per day by each rat was determined during the entire last experiment and at intervals during the pilot experiment. Each batch of diet was sampled from the germ-free cage. Each week aliquots of the combined collection of urine, feces, hair and wasted food from each rat were hydrolyzed for vitamin analysis.

Analyses were done by methods designed to give information about many vitamins from one sample. The method of hydrolysis is that recommended by Cheldelin et al. ('42) to obtain the highest values for B-vitamins in natural products. Most analyses were carried out by procedures described by Luckey et al. ('55). Citrovorum factor was determined using Leuconostoc citrovorum grown in the same medium as that

[3] Labco.

[4] Cellophane spangles, maximum particle size about 1 ml diameter. Rayon Processing Co., Central Falls, Rhode Island.

for the other B vitamins, using as the standard the calcium salt ($+5H_2O$) of a mixture of two diastereoisomers of leucovorin.

Inositol determinations were made using *Saccharomyces carlsbergensis* with adaptation of the method of Atkin et al. ('43), after autoclaving the enzymatic hydrolysate with 6 N sulfuric acid overnight.

As a check on our methods a sample was sent out f evaluation. We found one sample of dried rat feces to have 4.36 μg of folic acid per gram using the general enzymatic digestion. Dr. O. D. Bird of Parke Davis and Co. found it to have 3.87 μg of folic acid following 5-minute autoclaving and 7.22 μg of folic acid/gm after hog kidney conjugase treatment.

Whole blood clotting times were determined in capillary tubes. Prothrombin times were run by the diluted plasma method of Shapiro et al. ('42). Vitamin K activity was estimated using a chick bioassay.

RESULTS

The Caesarian-born, hand-fed germ-free rats used were quite small at weaning and grew somewhat more slowly than normally-born, dam-suckled germ-free rats. The growth curves (fig. 1) show that the weanling germ-free rats fed deficient diets (1-283 and L-295) grew at about the same rate as those fed the complete semisynthetic diet (L-109). Growth gave no clear indication of a requirement for biotin or folic acid. The conventional dam-reared rats grew faster than the germ-free rats fed the same diets. This may indicate the lack of a specific unknown factor, or it may possibly be attributed to the pre-weaning differences in diet or handling. Orland et al. ('54) report male conventional rats to be about 100 gm heavier than male germ-free rats after 150 days on experiment. A difference in rate of growth of female rats was not noticed.

Gross metabolism studies with rats fed semisynthetic diets indicate no obvious differences between germ-free and conventional rats. The germ-free rats ate and drank about the

same quantity as the conventional rats. The feces of the germ-free rats may be higher in moisture (25 to 30%) than those of conventional rats (20 to 25%), the fecal nitrogen is possibly higher in germ-free rats while the total quantity of fat excreted was very similar. Feces from germ-free rats have very few microorganisms present (these are, of course, dead cells introduced as transients in the autoclaved diet)

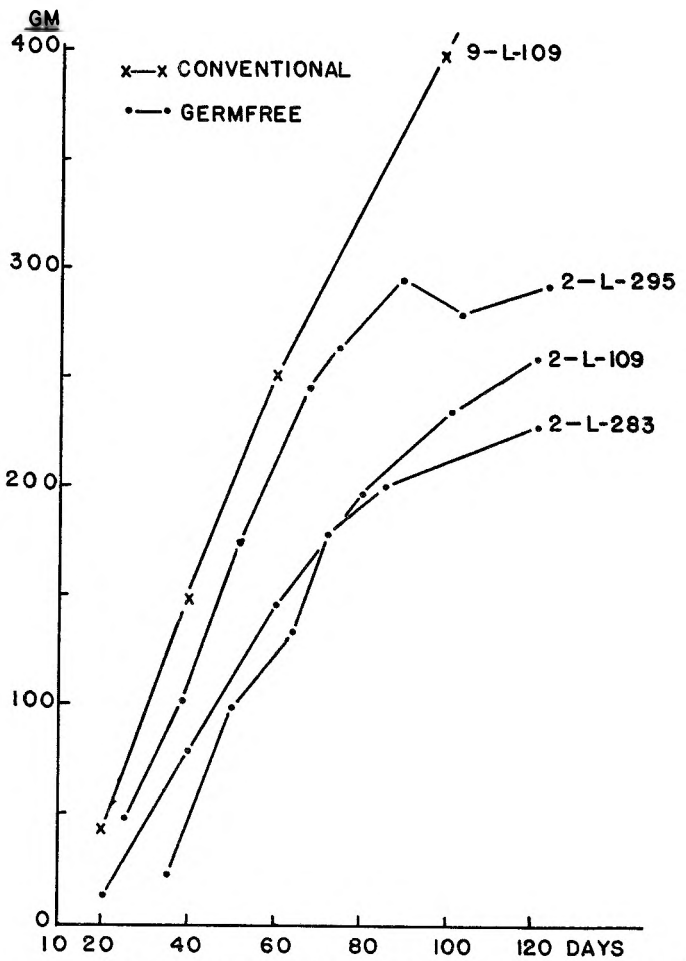

Fig. 1 Growth curves of male rats. The number of rats used and the diet designation is indicated. The germ-free rats fed diets L-283 and L-109 were Caesarian-born, hand-fed rats; the others were natural-born, dam-suckled rats.

while a substantial portion of the feces from conventional rats is microbial cells or their products. Details of morphology and organ analysis to be presented in a future publication indicate that the germ-free rats were similar to conventional rats in general appearance and gross composition (dry weight, ash, fat and nitrogen) of muscle, bone, spleen, liver, kidney and brain tissue from weaning to 9 months of age.

The blood picture of the germ-free rats may be summarized as follows: The total white counts were low (as is expected for germ-free rats) the differential counts were similar to those of conventional rats, and the hemoglobin values were 15.9 and 15.0 gm% for germ-free rats after 146 days on diet L-283 and 16.9 and 17.8 gm% for the conventional rats. At the end of the balance study one of the germ-free rats had 5.56×10^6 RBC/mm^3 with 13.2 gm% of hemoglobin and a normal differential count in both blood and bone smears.

Vitamin K studies. Germ-free rats fed semi-synthetic diets without added vitamin K showed no evidence of a vitamin K deficiency. Analysis of the diet for vitamin K indicated the presence of 2 to 10 μg of vitamin K per 100 gm of diet. The whole-blood clotting time for the two germ-free rats in the pilot experiment is given in minutes; 4, 4 at 186 days of age; 1, 1 at 192 days; 2.3 at 322 days; and 4.5 at 400 days. The prothrombin clotting time was 22 sec. (20 to 25 sec. for 5 runs) for the surviving germ-free rat at 400 days of age. No vitamin K could be detected in the liver, feces, bladder contents or cecal contents of this rat at 410 days. In the balance experiment the whole-blood clotting time of germ-free rats was 2.7 and 3.0 minutes after 163 days on diet 295.

Chicks fed a diet very similar to diet L-283 became deficient in vitamin K for a short time as evidenced by the whole-blood clotting time (Luckey, Pleasants and Reyniers, '55).

Biotin and folic acid. Germ-free rats fed diet L-283 (low in biotin and folic acid) gained weight continuously for the first 4 months (fig. 1). While the conventional rats continued to grow, the two germ-free rats began to lose weight (fig.

2) at 5 months of age and showed clinical symptoms of biotin deficiency which included weight loss, alopecia, spectacle eyes and open ulcers on shoulders, head and ears (fig. 3). One of the germ-free rats died after 152 days on the diet. The immediate cause of death appeared to be an intestinal blockage caused by levo-rotation of the enlarged cecum, a

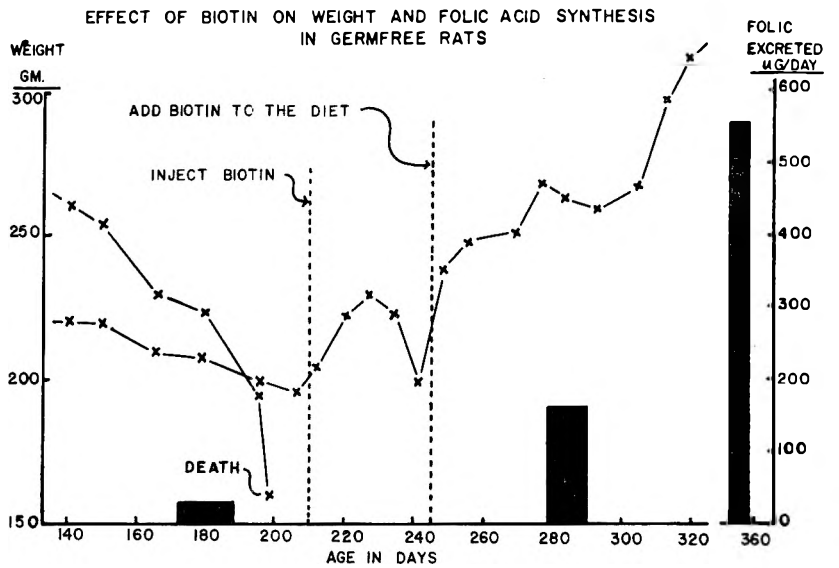

Fig. 2 Weight loss and response of individual germ-free rats deficient in biotin. The bars denote daily folic acid excretion.

DAYS	*Clinical observation*
140	In month preceding, some diarrhea in both rats: some loss of hair; eyes stuck shut with increasing frequency. Red exudate around eyes in one rat. Both rats had gained weight continuously for 140 days.
150	Anal area raw from diarrhea; hair coats thin with baldness on both sides of back. Skin on back dry and scaly.
157	Both eyes shut again in one rat.
177	Dry sores on shoulders.
185	Spectacle eyes with red exudate; sores on ears and "crusty' shoulder.
192	Pictures taken of rat No. 1.
198	Rat No. 1 dead.
210	General dermatitis and alopecia; biotin injected intraperitoneally. No weight.
222	Hair growing back; sores on face.
242	Biotin added to diet. No weight.
247	Hair growing; cataract in both eyes; new sores on head, shoulders and tail. General dermatitis clearing.
262	Sores healing; rat drinks and eats more than before.
271	Deep sore at base of tail.
290	Tail sore healing; shoulder sores bleeding.
311	Both shoulders raw.
317	Rat seems better; hair is growing.
385	Weight of rat is 404 gm.

common occurrence in hand-fed germ-free rats which has never been associated with any of the symptoms described above. The other rats were given 100 μg of biotin intra-muscularly. The conventional rats showed no reaction (they weighed 352 and 416 gm at this time) while the germ-free rat gave a remarkable growth response. New hair was seen growing but the ulcers on the head persisted. This quantity of biotin was apparently used up within three weeks and the rat again lost considerable weight. Biotin was then added to the diet and the rat continually gained weight during the

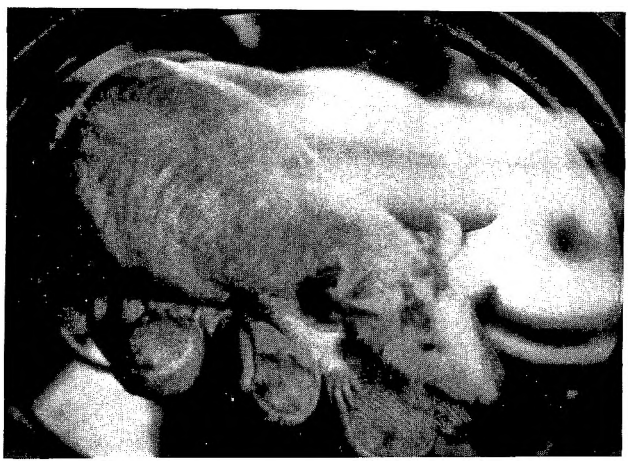

Fig. 3 Germ-free rat showing alopecia and ulcers from biotin deficiency. New growth of hair is seen on most of the body. The rat died one week later.

next 5 months (it weighed 404 gm when 385 days old). The ulcers appeared to heal when biotin was added to the diet; however they recurred sporadically.

Unexpectedly, when biotin was given to the surviving germ-free rat, the concentration of folic acid increased in both urine (from 1.47 to 4.91 μg/gm) and feces (from 8.79 to 28.0 μg/gm). This rat was eating about 25 gm of diet per day which contained 0.03 μg folic acid/gm as determined by microbiological assay. Since the folic acid concentration of the total rat carcass is less than 1 μg/gm, it would appear that this

rat could grow and at the same time excrete 100 to 500 μg of folic acid every day *only* by tissue synthesis of folic acid. Later, the folic acid in the feces of germ-free and conventional rats rose to such high concentrations (700 to 1,100 μg/gm) that an exceptionally active metabolite may be indicated.

In order to obtain further evidence on tissue synthesis of vitamins, a balance study was run. Four male germ-free rats were fed the diet with biotin added (diet L-295). Two rats were sacrificed after two weeks acclimatization. Analyses of their carcasses were used to estimate the quantity of vitamins present in the other two rats. Daily food consumption was recorded for the other two rats and all waste (feces, urine, hair and wasted food) was collected in a metabolism cage. Duplicate aliquots of the samples collected were analyzed about every two weeks. The animals were sacrificed for total carcass analysis 150 days after the beginning of the experiment.

The data summarized in table 1 indicate three states of vitamin balance. One, represented by niacin, shows that only a small proportion of the vitamin taken in was recovered. Other excretion products of niacin were expected but the study was necessarily limited to those forms of this vitamin that can be determined in the microbiological assay. Riboflavin and biotin were also incompletely recovered. It would appear that (1) forms of these vitamins exist which are inactive in the assay, or (2) some of these vitamins are catabolized in metabolic processes. Pantothenic acid and vitamin B_{12} appeared to be recovered in quantities approximately equal to the amounts supplied in the balance study by the weanling rat and the food. These equilibria do not exclude the possibility of biosynthesis of these vitamins. A relative rate of destruction, or the changing or binding of a vitamin into unmeasured forms which was faster than the rate of synthesis could give balance data which show only the apparent destruction. Thus only in the next group do the data give some assurance of a definite answer. The position of inositol is uncertain. The data in table 1 show a 50%

TABLE 1

Vitamin balance of germ-free rats fed diet L-295

		RAT	TOTAL WEIGHT	RIBO-FLAVIN	NIA-CIN	PANTO-THENATE	INOS-ITOL	BIOTIN	FOLIC ACID	CITRO-VORUM FACTOR	VITA-MIN B_{12}
			gm	*mg*	*mg*	*mg*	*mg*	*μg*	*μg*	*μg*	*μg*
Total input											
Weanling [1]		10	71.3	1.57	3.38	2.00	37.7	8.58	24.4	29.5	4.44
		12	109.2	2.27	4.42	3.03	59.1	5.87	44.6	36.6	4.65
		11	101.6	2.17	4.38	2.83	52.0	8.53	38.2	38.1	5.82
		13	101.0	2.16	4.36	2.81	51.6	8.48	38.0	37.8	5.79
Diet [2]		11	2783	99.8	258.2	594.4	472.4	2041	21.9	26.3	8.96
		13	2650	96.0	246.2	559.0	458.5	2076	21.0	26.0	8.92
Total output											
Waste		11	2416	62.8	68.0	537.8	683.7	1198	135.2	138.5	9.57
		13	2103	65.7	69.3	500.6	516.4	1502	227.4	285.9	10.50
Adult carcass		11	326.5	3.98	15.8	7.15	151.2	15.1	82.0	45.5	8.04
		13	237.2	2.63	7.35	5.27	110.5	13.4	107.0	36.3	4.07
Balance	In	11		102.0	262.6	597.3	524	2050	60.1	64.4	14.8
	Out	11		66.8	83.8	545.0	911	1213	217.2	183.9	17.6
	In	13		98.2	250.6	561.8	510	2085	59.0	63.8	14.7
	Out	13		68.3	76.6	505.9	713	1515	334	332	14.6
Output / Input $\times$ 100 [3]		11		63.8	31.7	91.4	174	59.2	361	286	119
		13		69.6	30.5	90.2	140	72.7	567	505	99.4
		Av.		66.7	31.1	90.8	157	65.0	464	396	109

[1] Initial vitamin data for rats 11 and 13 were calculated from body weight and the average vitamin data for rats 10 and 12.

[2] Calculated from individual analyses of 7 samples taken from the germ-free cage. Each sample was run in duplicate.

[3] The term $\frac{\text{output}}{\text{input}} \times 100$ gives a ratio of the amount of each vitamin recovered (from urine, feces, hair, wasted food and carcass at the end of experiment) to the amount put into the system (from the carcass at the start plus the diet).

increase over the input. The variation in inositol analyses of different lots of diet make it impossible to state unequivocally that inositol was synthesized. Three to 5 times more folic acid and citrovorum factor were recovered than was supplied initially by the rat and the diet. In the complete absence of any intestinal microbes this may be interpreted as tissue synthesis. These results confirm the results obtained in the pilot experiment.

DISCUSSION

The biotin deficiency exhibited by the germ-free rat is the exceptional example noted wherein the nutritional requirements of the germ-free animal differ qualitatively from those of the conventional animal. These experiments give direct evidence that the rat requires biotin from a source other than its own body stores and corroborates results obtained from feeding raw egg white (Bateman, '16; Boas, '27) or sulfa drugs (Black et al., '41, and Daft et al., '42). Speculation on the source of the daily requirement of the few micrograms of a micro-nutrient such as biotin supplied to the conventional rat (evidently unavailable to the germ-free rat) must include dust in the air, microbial synthesis in the water or food before ingestion, microbial synthesis in and absorption from the gastro-intestinal tract, and possibly tissue synthesis. Further direct experimentation on this question would be desirable.

Evidence for folic acid biosynthesis was seen in both experiments. In the first experiment the germ-free rats excreted no folic acid each day which was not accounted for in the food consumed; after biotin was added, the remaining germ-free rat was found to excrete about 50 times more folic acid than was taken in each day. This value was found to be greater two months later. Such high concentrations may indicate a "pseudo-folic acid." The balance experiment, run one year later, showed that citrovorum factor as well as folic acid was synthesized in two germ-free rats. The suggestion that biotin is required for folic acid biosynthesis in

the rat may clarify the interaction of these two vitamins in the sulfa-fed rat. A deficiency of folic acid might be anticipated any time the rat is involved in a biotin deficiency, unless the diet contains adequate quantities of one of the compounds in the folic acid group.

Since the compounds comprising the folic acid group and citrovorum factor apparently participate in the same vitamin function, the term vitamin "B_9" is suggested. The connotation of "vitamin B_9" would include all compounds having activity in the animal for folic acid and citrovorum factor but would be distinct from vitamin B_{12} activity (which includes vitamin B_{11} activity according to the work of Nichol et al., '49). The usefulness of a single term to designate these factors is readily seen. In this experiment we have considered the biosynthesis of "vitamin B_9" as measured by microbiological analyses for both folic acid and citrovorum factor. The true measurement might best be obtained by chick assay.

SUMMARY

Results from limited numbers of germ-free rats fed semisynthetic diets indicated that with an occasional exception the general metabolism of germ-free rats is very similar to that of conventional rats. Male germ-free rats do not grow as rapidly as do male conventional rats.

A dietary source of biotin is required by germ-free rats. The main symptoms of biotin deficiency were retarded growth, eventual loss of weight, alopecia, dermatitis and skin ulcers. Biotin was apparently required for the biosynthesis of folic acid.

A vitamin balance study in germ-free rats showed that less niacin, riboflavin and biotin were excreted and accumulated in body tissues than were fed to the rat; quantities of pantothenic acid and vitamin B_{12} excreted are equal to the amounts fed; biosynthesis of inositol was not proven unequivocally; while "vitamin B_9" (measured as folic acid and citrovorum factor) was synthesized.

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LITERATURE CITED

ATKIN, L., A. S. SCHULTZ, W. L. WILLIAMS AND C. N. FREY 1943 Yeast microbiological methods for determination of vitamins. Ind. Eng. Chem., Anal. Ed., *15:* 141–142.

BATEMAN, W. G. 1916 The digestibility and utilization of egg proteins. J. Biol. Chem., *26:* 263–291.

BLACK, S., J. M. MCKIBBON AND C. A. ELVEHJEM 1941 Use of sulfaguanidine in nutrition experiments. Proc. Soc. Exp. Biol. Med., *47:* 308–310.

BOAS, M. A. 1927 The effect of desiccation upon the nutritive properties of egg whites. Biochem. J., *21:* 712–724.

CHELDELIN, V. H., M. A. EPPRIGHT, E. E. SNELL AND B. M. GUIRAD 1942 Enzymatic liberation of B vitamins from plant and animal tissues. Univ. Texas Pub. 4237, p. 15–36.

COHENDY, M. 1912 Experiences sur la vie sans microbes. Ann. Inst. Pasteur, *26:* 106–137.

DAFT, F. S., L. L. ASHBURN AND W. H. SEBRELL 1941 Biotin deficiency and other changes in rats given sulfanilylguanidine or succinyl sulfathiazole in purified diets. Science, *96:* 321–322.

DU VIGNEAUD, V., C. RESELER, J. R. RACHELE, J. A. REYNIERS AND T. D. LUCKEY 1951 The synthesis of "biologically labile" methyl groups in the germ-free rat. J. Nutrition, *45:* 361–376.

GUSTAFSSON, B. 1946 Some experiences in germ-free rearing of rats. Nordisk Med., *32:* 2665–2666.

LUCKEY, T. D., J. R. PLEASANTS AND J. A. REYNIERS 1955 Germ-free chicken nutrition. II. Vitamin interrelationships. J. Nutrition, *55:* 105–118.

LUCKEY, T. D., J. A. REYNIERS, P. GYORGY AND M. FORBES 1954 Germ-free animals and liver necrosis. Ann. N. Y. Acad. Sci., *57:* 932–935.

NICHOL, C. A., L. S. DIETRICH, C. A. ELVEHJEM AND E. B. HART 1949 Folic acid deficiency in the chick in the presence of vitamin B_{12}. J. Nutrition, *39:* 387–398.

ORLAND, F. J., J. R. BLAYNEY, R. W. HARRISON, J. A. REYNIERS, P. C. TREXLER, M. WAGNER, H. A. GORDON AND T. D. LUCKEY 1954 Use of the germ-free animal technic in the study of experimental dental caries. I. Basic observation on rats reared free of all microorganisms. J. Dent. Res., *33:* 147–174.

PETERSON, W. H., AND M. S. PETERSON 1945 Relation of bacteria to vitamins and other growth factors. Bact. Rev., *9:* 49–109.

REYNIERS, J. A., P. C. TREXLER AND R. F. ERVIN 1946 Rearing germ-free albino rats. Lab. Rep., *1:* 1–86.

SHAPIRO, S., B. SHERWIN, M. REDISH AND H. A. CAMPBELL 1942 Prothrombin estimation: a procedure and clinical interpretations. Proc. Soc. Exp. Biol. Med., *50:* 85.

WILLIAMS, R. J., R. B. EAKIN, E. BEERSTECKER, JR. AND W. SHIVE 1950 The biochemistry of B vitamins, Reinhold Pub. Corp., New York.

NUTRITIONAL INTAKE OF CHILDREN

III. THIAMINE, RIBOFLAVIN AND NIACIN [1]

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(Received for publication April 23, 1955)

TWO FIGURES

INTRODUCTION

During the years of growth the dietary intake of a child may be affected by a number of factors. Physiologic demand for the building and maintenance of body tissues constitutes the major factor, one which varies with age and developmental status but is not yet well understood or evaluated. Interrelated environmental and intrinsic factors may alter intake or utilization. For example, appetite may be increased or decreased by the emotional changes within the child himself as he reacts to the progress of his own maturation and to the people and events in his surroundings. As a result there are changes in the level of intake as the individual progresses from birth to maturity; the significance of these changes in regard to both cause and effect presents a challenging field of study.

This series of papers is intended to present the picture of nutritional intake during the first 5 years of life by a group of healthy children living at home.

METHODS OF STUDY

The methods employed in this study were reported in detail in a previous publication (Beal, '53). Nutrition studies

[1] This study was aided in part by a grant from the Nutrition Foundation.

were added in 1946 to the program of the Child Research Council, which has been engaged in research on growth and development for more than 25 years. The children, who come from "upper middle class" families in the Denver area, have been voluntarily enrolled in this study by their parents; no effort is made by the Council staff to influence dietary intake.

The nutrition data are based on a series of histories obtained by interview during home visits, checked by concomitant 24-hour intakes recorded by the nutritionist and by the mother. Histories and intakes are obtained at intervals of one month during the first 6 months of life and thereafter at intervals of three months. Nutrients are calculated from food value tables (Bowes and Church, '51; U. S. Department of Agriculture, '48 and '50).

The data in this paper represent 934 histories on 63 children (29 boys and 34 girls) who now range in age from 5 months to 11 years. Only the first 5 years of life are included. Excluded from these data are histories of the intake of breast-fed infants during the period of such feeding and two single histories on older children in whom severe illness resulted in a marked decrease in food intake during an entire three-month period. All other histories on these 63 children during this age span have been included.

Intakes of calories, carbohydrate, fat, protein and three minerals have already been reported (Beal, '53 and '54). The present publication is concerned with the levels of thiamine, riboflavin and niacin in the foods consumed. Sixty-eight per cent of the children in this series have taken supplementary vitamin preparations containing the B complex for varying periods of time; an average of 40% of the histories at each age include such preparations. Since there is no significant difference in height, weight or calorie intake of the children receiving supplements of these three vitamins as compared with children who did not receive such supplements, the data presented will be concerned solely with the intakes of these vitamins from foods.

RESULTS AND DISCUSSION

As previously reported, the intake of all nutrients for which calculations have been made rises rapidly and relatively smoothly during the first 9 months of life. This is a period of rapid growth and, for most children, a period of relative freedom from problems associated with eating. Appetite is usually good and there is a steady rise in food consumption as milk intake is increased and new foods are added to the dietary. Toward the end of the first year many changes may be observed, with a decrease in growth rate and an acceleration of social and motor development. There is an alteration in the method of feeding, with progression toward independence, self-feeding, expression of choice, and replacement of soft solids and especially prepared infant foods with those of coarser texture. There are marked changes in acceptance of specific foods. As a result, intake levels of calories, carbohydrate and fat increase only slightly between one and three years, protein intake remains stationary, and intakes of calcium, phosphorus and iron drop to a low point between two and three years. After three years of age, intake of all these nutrients rises.

Intakes of thiamine and riboflavin of the children in this study from birth to 5 years of age are presented in table 1. Because these data do not show a normal distribution but instead tend to be skewed to the right, percentiles have been used as being more accurately descriptive than means and standard deviations. The table gives the values derived from visual smoothing of the curves of intake at the levels of the 25th, 50th and 75th percentiles; lowest and highest intakes observed to date are also shown.

The curve of intake of thiamine has a configuration similar to the curve of protein intake. After a steady rise in the first 15 months, a plateau is reached which is maintained until just after three years, so that the three-year-old child is consuming no more protein or thiamine than at 15 months. After three years of age, thiamine intake shows a steady increase. Except at one to three months, the Recommended

TABLE 1

Thiamine and riboflavin intake of children from birth to 5 years of age

AGE	NO. OF CASES	THIAMINE Percentile					RIBOFLAVIN Percentile				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
years *months*		*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
0–0 to 0–1	32	0.07	0.11	0.12	0.18	0.55	0.25	0.71	0.82	0.93	1.35
0–1 to 0–2	37	0.09	0.15	0.17	0.23	0.48	0.37	0.95	1.11	1.33	1.57
0–2 to 0–3	38	0.12	0.19	0.22	0.30	0.56	0.41	1.07	1.29	1.49	1.80
0–3 to 0–4	41	0.18	0.22	0.27	0.39	0.60	0.41	1.18	1.37	1.57	2.00
0–4 to 0–5	43	0.19	0.28	0.37	0.49	0.80	0.46	1.27	1.44	1.65	2.11
0–5 to 0–6	44	0.28	0.35	0.45	0.57	1.01	0.50	1.35	1.51	1.72	2.28
0–6 to 0–9	47	0.32	0.44	0.56	0.67	0.88	1.09	1.46	1.59	1.81	2.38
0–9 to 1–0	49	0.25	0.53	0.67	0.76	1.10	1.05	1.50	1.64	1.88	2.47
1–0 to 1–3	47	0.40	0.58	0.70	0.80	1.02	0.92	1.48	1.64	1.88	2.38
1–3 to 1 6	47	0.44	0.58	0.70	0.80	1.10	1.06	1.40	1.57	1.82	2.61
1–6 to 1–9	44	0.38	0.58	0.70	0.80	1.19	0.76	1.29	1.46	1.72	2.66
1–9 to 2–0	45	0.38	0.58	0.70	0.80	1.07	0.72	1.22	1.40	1.67	2.34
2–0 to 2–3	44	0.41	0.58	0.70	0.80	1.10	0.65	1.17	1.38	1.64	2.35
2–3 to 2–6	42	0.34	0.58	0.70	0.80	1.23	0.87	1.14	1.37	1.62	2.30
2–6 to 2–9	38	0.39	0.58	0.70	0.80	1.17	0.52	1.12	1.36	1.62	2.34
2–9 to 3–0	38	0.39	0.58	0.70	0.80	1.20	0.70	1.12	1.36	1.62	2.28
3–0 to 3–3	36	0.42	0.59	0.70	0.80	1.20	0.71	1.14	1.38	1.63	2.58
3–3 to 3–6	35	0.29	0.60	0.71	0.81	1.11	0.67	1.16	1.40	1.66	2.29
3–6 to 3–9	35	0.42	0.61	0.72	0.82	1.04	0.68	1.20	1.44	1.70	2.16
3–9 to 4–0	32	0.35	0.62	0.74	0.84	0.98	0.59	1.24	1.48	1.75	2.19
4–0 to 4–3	34	0.31	0.64	0.75	0.86	1.35	0.90	1.28	1.53	1.80	2.40
4–3 to 4–6	30	0.38	0.66	0.77	0.88	1.24	0.73	1.34	1.59	1.85	2.58
4–6 to 4–9	28	0.42	0.67	0.79	0.90	1.09	0.85	1.39	1.65	1.90	2.38
4–9 to 5–0	28	0.50	0.69	0.81	0.92	1.16	0.90	1.45	1.72	1.96	2.25

Dietary Allowance of the National Research Council ('53) falls just below the median of these data.

Expressed in terms of caloric intake, the thiamine median rises from a level just above 0.30 mg per 1000 Cal. in the first two months to approximately 0.70 mg per 1000 Cal. by one year, then decreases in the next two years to a level of 0.54 mg per 1000 Cal., a level which is maintained from three to 5 years. The decrease observed between one and three years of age is a reflection of the gradual increase in total calories during the period when thiamine intake remains unchanged.

Thiamine intakes of individual children show a remarkable constancy of position in the group. The most common pattern is that of stability within one quartile range throughout most of this period.

In contrast, riboflavin intake, after an initial rise in the first year, decreases as does the calcium intake during the second and third years when milk consumption decreases, then begins to rise between three and 4 years. The intakes of individual children show much more lability in riboflavin than in thiamine. In figure 1, intakes of three individual children are plotted against the 25th, 50th and 75th percentiles of the group to show the differences in patterns of intake. One child, after the first 6 months, maintains a level usually near the top of the group. The intake of the second child is low during the first year but thereafter is close to the median. The intake of the third child drops from an initially high level in the first 5 months to a position in the lowest quartile between three and 5 years. In each case the graph of riboflavin intake closely parallels the graph of milk intake. There is no significant correlation in this group between riboflavin intake, calculated in terms of the daily average, and plasma riboflavin determinations (Bartlett, '55).

The Recommended Dietary Allowances for riboflavin are indicated in figure 1. Throughout this age span the Allowance is below the 25th percentile, approaching the minimum of this group. While it is possible that these children have an

intake of riboflavin which is well above their requirement, even during the preschool years when milk consumption is low, there is increasing evidence of a relationship between the protein requirement and the riboflavin requirement and of greater retention of riboflavin during periods of nitrogen storage (Pollack and Halpern, '52). It is not inconceivable, then, that the need for riboflavin during periods of growth may be greater than has heretofore been recognized.

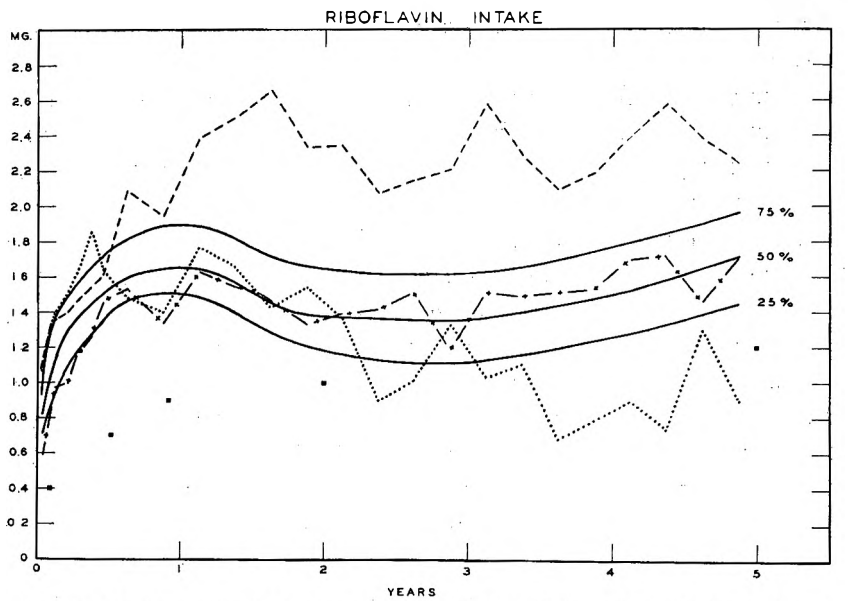

Fig. 1 Patterns of riboflavin intake of three children from birth to 5 years of age, plotted against the 25th, 50th and 75th percentiles of the group. N. R. C. Recommended Allowances are indicated by the black squares.

Percentile levels of niacin intake are presented in table 2. In comparison to the nutrients thus far discussed, in which the 25th, 50th and 75th percentiles tend to be parallel to each other after the first year, the curves of niacin intake show a striking contrast from one to three years between the higher and lower levels. While the 25th percentile reaches a plateau during the second year, the median continues to rise, but with a sharp break in the second year in the steep-

ness of the curve, and the 75th percentile continues to rise during the second year and shows a drop in the third year. The percentile curves tend to be parallel to each other between three and 5 years. Of the entire group of children who have passed through this age span, approximately one-

TABLE 2

Niacin intake of children from birth to 5 years of age

AGE	NO. OF CASES	NIACIN Percentile				
		Lowest	25	50	75	Highest
years *months*		*mg*	*mg*	*mg*	*mg*	*mg*
0–0 to 0–1	32	0.3	0.4	0.5	0.6	1.7
0–1 to 0–2	37	0.4	0.6	0.7	0.9	2.9
0–2 to 0–3	38	0.5	0.7	0.9	1.2	2.7
0–3 to 0–4	41	0.6	0.9	1.3	1.7	4.0
0–4 to 0–5	43	0.6	1.2	1.8	2.3	5.7
0–5 to 0–6	44	1.1	1.6	2.3	2.9	7.8
0–6 to 0–9	47	1.6	2.5	3.1	4.0	6.5
0–9 to 1–0	49	1.1	3.5	4.1	5.2	10.4
1–0 to 1–3	47	2.2	3.9	4.8	6.0	10.3
1–3 to 1–6	47	2.6	4.1	5.2	6.7	12.3
1–6 to 1–9	44	2.6	4.1	5.4	7.2	12.8
1–9 to 2–0	45	2.1	4.1	5.5	7.6	13.6
2–0 to 2–3	44	2.1	4.2	5.5	7.6	15.3
2–3 to 2–6	42	2.3	4.3	5.6	7.5	14.1
2–6 to 2–9	38	2.6	4.4	5.7	7.1	14.9
2–9 to 3–0	38	2.7	4.5	5.8	7.0	13.6
3–0 to 3–3	36	3.5	4.6	5.9	7.0	11.3
3–3 to 3–6	35	2.1	4.7	6.0	7.2	10.7
3–6 to 3–9	35	3.0	4.8	6.1	7.3	11.5
3–9 to 4–0	32	2.7	5.0	6.2	7.5	9.9
4–0 to 4–3	34	3.8	5.2	6.4	7.7	11.0
4–3 to 4–6	30	3.3	5.4	6.6	7.9	11.7
4–6 to 4–9	28	3.7	5.6	6.9	8.1	13.6
4–9 to 5–0	28	3.7	5.8	7.1	8.3	13.0

fourth show a niacin decrease in the third year. If, however, only those children whose intake was above the median in the second year are considered, more than half show a decrease in the third year. This is readily explainable by an analysis of the data on meat intake. The children who had a small

intake of meat during the second year tended not to decrease their intake during the third year, while the children at a higher level of intake did show this subsequent decrease.

Examples of this change in niacin intake are demonstrated by the three children shown in figure 2. While the intakes of these three children are not too dissimilar near the end of the first year, both children whose intake is above the median in the second year show a decrease by the end of the

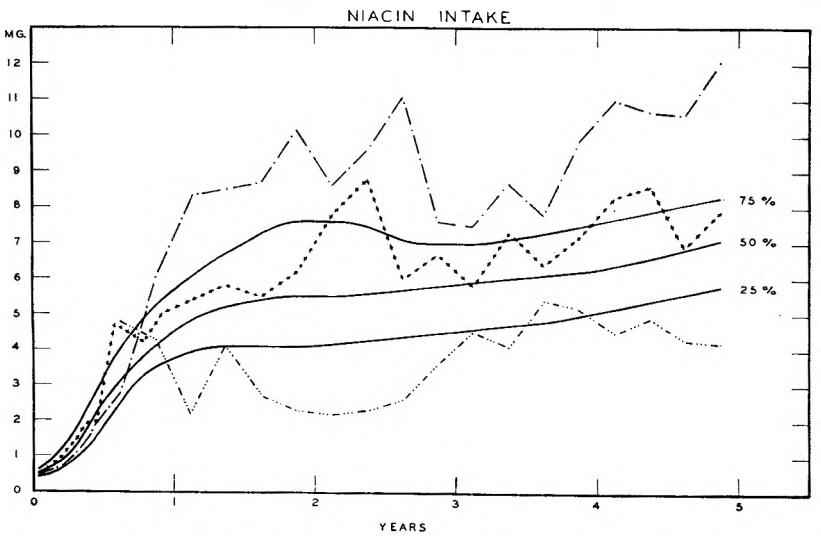

Fig. 2 Patterns of niacin intake of three children from birth to 5 years of age, plotted against the 25th, 50th and 75th percentiles of the group.

third year. The third child (whose intake during the first 6 months is not shown because of breast feeding) dropped to a level below the 25th percentile early in the second year with the change from canned strained meats to home-cooked meats, and tended to maintain that low level until 5 years of age. There is no significant correlation between niacin intake and red cell levels of niacin of the children in this study.

The Recommended Dietary Allowances for niacin are above the 75th percentile level of this group in the first year and

just below the 75th percentile thereafter. Although the niacin intake of the children enrolled in this study tends to be low when compared with the Allowances, no evidences of niacin deficiency have been observed, even among the 32% who have not received niacin supplements. It must be kept in mind that the diets of these children are well supplied with protein and, although no attempt has yet been made to assess the amino acid content, it may be assumed that adequate amounts of tryptophan are being supplied. The level of niacin intake observed in the diets of these children, then, seems adequate.

SUMMARY

Data have been presented from 934 nutrition histories on 63 children in the first 5 years of life. Thiamine, riboflavin and niacin intakes have been computed in terms of quartiles and maximum and minimum levels observed. Some individual patterns of intake are shown.

The intake of thiamine rises steadily during the first 15 months, then reaches a plateau which is maintained until just after three years, after which it increases again. The median of these data is slightly above the National Research Council Recommended Allowance.

The riboflavin intake, after an initial rise in the first year, decreases in the second and third years with a decrease in milk consumption, then rises again between three and 5 years. More than 75% of the children in this group consume an amount of riboflavin greater than the Recommended Allowance throughout this age period.

The niacin intake tends to increase throughout this age span, although the children with high intakes during the second year are likely to show a decrease during the third year. Although only 25% of the children meet the Recommended Allowance, there is no evidence that their intake is inadequate, as judged by growth rate and absence of deficiency symptoms.

LITERATURE CITED

BARTLETT, M. 1955 Red blood cell niacin and plasma riboflavin levels in a group of normal children. J. Nutrition, *57:* 157.

BEAL, V. A. 1953 Nutritional intake of children. I. Calories, carbohydrate, fat and protein. Ibid., *50:* 223.

———— 1954 Nutritional Intake of Children. II. Calcium, phosphorus and iron. Ibid., *53:* 499.

BOWES, A. DE P., AND C. F. CHURCH 1951 Food Values of Portions Commonly Used. College Offset Press, Philadelphia, Pa., 7th ed.

NATIONAL RESEARCH COUNCIL 1953 Recommended Dietary Allowances. Publication 302.

POLLACK, H., AND S. L. HALPERN 1952 Therapeutic Nutrition. National Academy of Sciences — National Research Council Publication 234. (Riboflavin, page 34.)

UNITED STATES DEPARTMENT OF AGRICULTURE 1948 Vitamin and Mineral Content of Certain Foods as Affected by Home Preparation. Washington, D. C. Miscellaneous Publication No. 628.

———— 1950 Composition of Foods — Raw, Processed, Prepared. Agriculture Handbook No. 8.

STARCHES, SUGARS AND RELATED FACTORS AFFECTING LIVER FAT AND NITROGEN BALANCES IN ADULT RATS FED LOW LEVELS OF AMINO ACIDS

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It has been established that fat accumulates in the livers of rats fed low levels of amino acids or proteins with sucrose as the carbohydrate even when choline is present in the rations (Harper et al., '53a; Singal et al., '53; Marshall and Womack, '54). When corn dextrin was substituted for sucrose, liver fat approached normal levels and nitrogen balances of adult rats (Marshall and Womack, '54) or the growth rates of young animals (Litwack et al., '52) were improved. Substitution of glucose for sucrose (Harper et al., '53b) in low protein rations, or supplementation with threonine (Harper et al., '53a; Singal et al., '53) increased the growth rate and reduced liver fat, while the effect of fructose in the diet was similar to that of sucrose (Harper et al., '53b). A recent report by Shils et al. ('54) indicates that young rats fed diets consisting largely of corn meal, rice, wheat or cassava developed portal type of fatty livers said to bear a similarity to the type of fatty liver seen in the human disease kwashiorkor.

In the present studies, the comparative effects of several starches on the accumulation of liver fat and on nitrogen balance in adult protein-depleted rats fed low levels of amino acids have been investigated. In addition, the effects of

related factors, such as feeding glucose and fructose, supplementation with selected amino acids and vitamins, addition of cellulose and of sulfasuxidine, and use of a different strain of rats and feeding management, were tested to aid in interpretation of the findings.

EXPERIMENTAL

The nitrogen-free diet consisted of a source of carbohydrate, 80.65%; salt mixture (Jones and Foster, '42), 4%; corn oil, 3%; lard, 12%; vitamin A and D concentrate [1], 0.05%; inositol, 0.1%; and choline chloride, 0.2%. The following vitamins were added per kilogram of ration: thiamine HCl, 5 mg; pyridoxine HCl, 5 mg; niacin, 5 mg; riboflavin, 10 mg; Ca pantothenate *(d)*, 25 mg; *p*-aminobenzoic acid, 300 mg; α-tocopherol acetate, 25 mg; 2-methyl-1, 4-naphthoquinone, 2 mg; folic acid, 2 mg; biotin, 100 µg; and vitamin B_{12}, 30 µg. Additions were made to the diets at the expense of the carbohydrate. The compositions of the amino acid mixtures have been described (Womack et al., '53). Amino acid mixture 1 contained 0.1% and mixture 2, 0.2% of each of the essential amino acids. Both mixtures contained 0.1% of each of the non-essential amino acids. The control diet contained mixture 1 with sucrose as the carbohydrate.

Corn, potato, rice and wheat starches [2] were cooked under standardized conditions. Two kilograms of starch were suspended in two liters of cold water and added, with stirring, to 7 l of boiling water in a steam-jacketed kettle. In addition, one liter of water was used to rinse the starch container. The mixture was covered and cooked for 10 minutes, spread in thin layers in pans and dried in a dehydrator at room temperature. The dried starches were ground in a ball mill to pass through a 20-mesh sieve.

[1] Squibb's Navitol containing 65,000 U. S. P. units of vitamin A and 13,000 U. S. P. units of vitamin D per gram.

[2] Purchased from Morningstar, Nicol, Inc.

Male rats from the laboratory stock colony [3], or (in experiment VII) from a strain of Wistar rats [4], were housed individually and fed the commercial stock ration ad libitum from weaning until 91 to 105 days old. They were divided into groups according to litter and body weight. Animals receiving the nitrogen-free rations were fed ad libitum, as were those of groups 37 and 38. All other animals were offered 12 gm of food (4.6 calories per gm) per day. Nitrogen balances were determined during the last 7 days of the 18-day protein-depletion period and, after a week of adjustment, during two consecutive 7-day periods of amino acid feeding. Weekly weighings were made of the animals and of their uneaten food. The methods of collecting the urine and feces and of the nitrogen analyses have been previously described (Womack et al., '53; Marshall and Womack, '54).

Seven experiments were conducted. In experiment I, 7 groups of animals were depleted of protein on nitrogen-free diets containing either sucrose, corn dextrin [5], raw cornstarch, or one of the 4 cooked starches, then given corresponding diets with amino acid mixture 1 added. All animals of experiments II to VII, except groups 16 and 36, were depleted of protein on nitrogen-free diets containing sucrose and then fed the various diets indicated in table 1. Group 16 received wheat starch and group 36 corn dextrin during the depletion period.

After completion of the nitrogen balance studies, the livers of the animals were rapidly removed under amytal anesthesia and preserved for determination of fat, protein and moisture content, as previously described (Marshall and Womack, '54). For some of the groups, a selected lobe of the liver was divided into two samples which were placed immediately in 30%

[3] A purebred strain resulting from the crossing of albino and black and white hooded strains of *mus norvegicus*. The litters may include white, black, and black and white rats.

[4] Kindly supplied by Dr. Leo Friedman of the Food and Drug Administration, Washington, D. C.

[5] Amidex, from Corn Products Refining Company.

potassium hydroxide solution and the glycogen content determined with anthrone by the method of Seifter et al. ('50).

RESULTS AND DISCUSSION

Analysis of variance was used to aid in evaluation of the results. Statistically significant decreases in the fat content of the liver resulted from feeding corn dextrin, raw or cooked cornstarch, cooked potato starch, cooked rice starch or cooked wheat starch (table 1, experiments Ib, II, III and VII) or glucose (group 19, experiment IV) instead of sucrose in diets containing low levels of amino acids. The addition of 0.2% of DL-threonine (groups 21 and 29) or 0.1% of DL-threonine (group 33) to the control diet, or doubling the concentration of the essential amino acids (group 24) decreased liver fat to normal or near normal levels. Additions to the sucrose-containing rations of 2% of cellu flour (group 17), 100 mg of niacin per kilogram (group 28), 0.1% of DL-methionine (group 30) or 1% of sulfasuxidine (group 32) brought about no significant changes in liver fat.

Animals fed fructose (group 20) had as much fat in the livers as those fed sucrose (group 18). Addition of 0.2% of DL-threonine (group 23) or substitution of 0.2 for 0.1% of the essential amino acids (group 26) reduced liver fat of the animals receiving fructose almost to normal values. From these results it would appear that in the use of fructose in diets of diabetic patients (Miller et al., '52; Smith et al., '53) care should be taken to ensure the adequacy of the amino acid intake, or at least threonine intake, to preclude the possibility of the development of fatty livers.

Average daily negative nitrogen balances were decreased although not always significantly in the same groups in which the liver fat was reduced with the exception of the animals receiving potato starch (groups 7b and 13) and glucose (group 19) [6]. Differences required for significance between

[6] Although the balances of group 21 were not significantly different from those of group 18, the nitrogen balances of group 18 were considerably lower than those of other groups receiving the control diet.

the means are given in table 1. No significant differences were observed in the nitrogen balances or in the liver fat between groups of animals fed the same starches in experiments Ib (depleted on starches or corn dextrin) and II (depleted on sucrose). In both experiments, rice starch caused the greatest improvement in the balances. In experiment III, nitrogen balances and liver fat of the two groups of animals receiving wheat starch, one depleted on starch, the other on sucrose, were not different from each other. Therefore, despite differences in nitrogen balances and in liver fat between the sucrose-fed groups (1b and 8), the performance of the animals fed the various starches in experiments Ib and II does not appear to be influenced by the carbohydrate fed during the depletion period.

In experiment VII in which the performance of another strain of rats (Wistar) was studied, groups 37 and 38 were allowed to eat ad libitum. With increased food intake the negative nitrogen balances, but not the liver fat, were reduced. Whether the effect on the nitrogen balances was due to increased amino acid or calorie intake, or to increased intake of some other nutrient cannot be decided from these studies.

Nitrogen balances of depleted animals fed sucrose or corn dextrin with amino acid mixture 1, except for groups 8 and 18, were considerably more negative than those previously reported for somewhat smaller animals fed the same diets (Womack et al., '53; Marshall and Womack, '54). Nitrogen balances during periods of protein depletion are known to be related to body size. During the last 7 days of the 18-day protein-depletion period of this study, daily nitrogen balances averaging — 58.6 mg were found for 68 animals whose weight was 330 to 339 gm. The nitrogen balances of 33 animals whose initial weight was 370 to 379 gm averaged — 67.3 mg. During the periods when amino acid mixture 1 was fed with sucrose, average daily nitrogen balances of — 11.9 mg were found for 21 animals whose weight before depletion was 330 to 339 gm and of — 19.5 mg for 7 animals whose initial weight

TABLE 1

Average daily nitrogen balances and liver composition of protein-depleted adult male rats fed low levels of amino acids and various sources of carbohydrates, with or without certain other supplements

GROUP NO.	DESCRIPTION OF DIET [1]	BODY WEIGHT		INTAKE		N EXCRETION		N BALANCE IN N-FREE PERIOD [2]	NITROGEN BALANCE AND STANDARD ERROR [2]	TEST DIFFERENCE [3]	LIVER			
		Initial	Final	Food	N	Feces	Urine				Weight [2]	Protein [4]	Glycogen [2,4]	Fat and standard error [4]
		gm	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*		*gm*	%	%	%
	Stock [5]										12.72 (14)	21.3	4.2 (10)	2.2 ± 0.1
	Experiment Ia — Nitrogen-free													
1a	Sucrose	360	302	10.6	2.1	11.8	57.8		— 67.5 ± 1.9 (12)		9.90 (15)	14.8	8.9 (5)	6.7 ± 0.7
2a	Corn dextrin	359	308	12.9	8.5	18.0	50.4		— 59.9 ± 3.1 (9)		9.86 (6)	15.4		4.9 ± 0.8
3a	Cornstarch, raw	361	318	12.6	6.3	13.7	54.0		— 61.4 ± 1.4 (11)		9.65 (6)	14.7		7.1 ± 1.9
4a	Cornstarch, cooked	366	324	12.9	6.4	22.9	48.0		— 64.4 ± 1.9 (11)		8.87 (6)	15.4		5.0 ± 0.4
5a	Rice starch, cooked	356	319	11.3	9.1	21.0	46.2		— 58.2 ± 3.2 (15)		9.23 (7)	14.8		5.2 ± 0.9
6a	Wheat starch, cooked	348	301	9.3	8.0	20.8	44.3		— 57.1 ± 1.2 (11)		8.17 (4)	15.6		3.9 ± 0.5
7a	Potato starch, cooked	349	301	11.0	3.3	25.6	39.4		— 61.7 ± 1.6 (13)		8.74 (5)	14.8		5.3 ± 1.2
	Experiment Ib — 0.1% EAA + 0.1% NEAA [6]													
1b	Sucrose	359	294	11.4	35.0	11.7	41.0	— 67.5 (12)	— 17.7 ± 1.3 (17)	7.0	12.13 (17)	13.5		16.5 ± 1.1
2b	Corn dextrin	355	293	11.7	42.0	15.8	37.3	— 59.5 (7)	— 11.0 ± 1.6 (7)		8.58 (7)	16.7		6.9 ± 0.6
3b	Cornstarch, raw	366	309	11.2	38.2	13.0	36.4	— 61.4 (8)	— 11.2 ± 1.0 (8)		10.30 (10)	15.2		9.5 ± 1.1
4b	Cornstarch, cooked	371	315	11.7	38.5	20.1	31.2	— 64.7 (8)	— 12.7 ± 1.1 (8)		9.83 (11)	15.2		7.1 ± 0.7
5b	Rice starch, cooked	356	309	11.7	42.0	19.4	30.6	— 58.6 (8)	— 8.0 ± 1.5 (8)		10.00 (11)	15.3		7.6 ± 1.0
6b	Wheat starch, cooked	352	297	12.0	43.2	23.4	31.7	— 58.1 (7)	— 11.8 ± 1.2 (7)		9.28 (10)	15.9		9.0 ± 1.2
7b	Potato starch, cooked	350	292	12.0	37.9	25.9	28.2	— 61.6 (8)	— 16.2 ± 0.5 (8)		9.20 (11)	15.3		7.3 ± 1.2
	Experiment II — 0.1% EAA + 0.1% NEAA													
8	Sucrose	352	302	11.8	37.0	11.4	39.0	— 62.1 (8)	— 13.3 ± 0.8 (8)	3.5	15.46 (8)	11.9	4.4	26.2 ± 1.8
9	Corn dextrin	360	303	11.7	41.2	14.2	37.1	— 60.5 (8)	— 10.1 ± 0.8 (8)		11.25 (8)	15.4	6.7	8.3 ± 1.4
10	Cornstarch, cooked	364	309	12.0	39.3	19.9	31.2	— 63.4 (8)	— 11.7 ± 0.9 (8)		11.14 (8)	14.4	9.5	7.5 ± 1.1
11	Rice starch, cooked	360	305	11.8	43.1	19.1	33.1	— 62.1 (8)	— 9.2 ± 0.9 (8)		11.13 (8)	14.7	7.4	10.7 ± 2.9
12	Wheat starch, cooked	358	306	12.0	43.0	19.5	34.4	— 61.9 (8)	— 10.8 ± 0.9 (8)		11.16 (8)	14.4	9.3	8.5 ± 1.5
13	Potato starch, cooked	361	303	11.9	38.2	24.8	28.1	— 61.6 (8)	— 14.6 ± 0.8 (8)		10.23 (8)	14.5	10.0	6.5 ± 0.7
	Experiment III — 0.1% EAA + 0.1% NEAA													
14	Sucrose	359	298	11.7	36.3	13.3	44.8	[7]	— 21.8 ± 1.9 (5)		13.60 (5)	13.2	6.0	18.9 ± 2.4
15	Wheat starch, cooked	355	300	12.0	44.7	22.9	36.4	— 67.8 (5)	— 14.7 ± 1.0 (6)		11.90 (6)	13.9	9.3	10.5 ± 1.5
16	Wheat starch, cooked [1]	357	303	12.0	44.7	21.9	37.0	— 70.4 (6)	— 14.2 ± 0.9 (6)		10.83 (6)	15.2	8.9	7.3 ± 1.4
17	Sucrose + 2% cellu flour	359	297	11.9	36.9	13.3	43.0	— 66.5 (6)	— 19.4 ± 1.4 (6)		15.63 (6)	12.3	5.5	23.0 ± 1.3

	Experiment IV — 0.1% EAA + 0.1% NEAA												
18	Sucrose	349	299	11.4	36.0	10.3	36.6	— 56.8 (7)	— 10.9 ± 1.2 (7)	5.0	13.19 (7)	12.8	6.0	19.4 ± 1.3
19	Glucose	345	284	11.1	34.3	9.2	38.4	— 55.9 (7)	— 13.3 ± 1.1 (7)		9.50 (7)	15.9	5.8	10.4 ± 1.3
20	Fructose	341	288	11.4	35.8	10.5	42.5	— 52.9 (7)	— 17.2 ± 1.1 (7)		13.45 (7)	12.7	6.2	19.3 ± 1.9
21	Sucrose + 0.2% DL-threonine	356	298	10.2	33.7	10.1	33.3	— 62.2 (7)	— 9.6 ± 0.7 (7)	4.0	9.61 (7)	15.9	7.1	7.4 ± 0.9
22	Glucose + 0.2% DL-threonine	357	286	9.0	30.5	7.8	30.4	— 59.0 (6)	— 7.7 ± 0.8 (6)		8.10 (6)	17.0	7.2	4.1 ± 0.4
23	Fructose + 0.2% DL-threonine	355	291	9.3	30.8	9.6	33.8	— 57.4 (7)	— 12.5 ± 1.2 (7)		9.95 (7)	15.4	8.2	5.7 ± 0.5
	Experiment V — 0.2% EAA + 0.1% NEAA												
24	Sucrose	359	306	11.9	54.8	12.0	41.3	— 66.0 (6)	+ 1.5 ± 1.9 (6)	4.5	10.96 (6)	15.6	8.9	5.6 ± 0.7
25	Glucose	358	304	11.3	53.3	10.4	37.0	— 59.9 (7)	+ 5.9 ± 0.9 (7)		9.48 (7)	16.9	7.2	4.8 ± 0.5
26	Fructose	355	299	11.3	54.3	10.6	42.5	— 62.1 (7)	+ 1.2 ± 1.3 (7)		11.51 (7)	14.9	9.2	6.9 ± 0.7
	Experiment VI — 0.1% EAA + 0.1% NEAA												
27	Sucrose	359	289	11.6	35.4	12.2	43.0	[7]	— 19.8 ± 2.8 (6)	6.0	11.85 (6)	14.0		14.7 ± 1.9
28	+ 100 mg/kg niacin	363	297	11.7	35.2	11.6	40.6	— 68.1 (6)	— 17.0 ± 0.8 (8)		12.39 (8)	13.4		18.9 ± 1.1
29	+ 0.2% DL-threonine	369	286	10.3	33.9	10.5	36.0	[7]	— 12.6 ± 0.7 (8)		9.38 (8)	16.5		4.2 ± 0.4
30	+ 0.1% DL-methionine	366	295	11.3	35.3	11.1	40.5	[7]	— 16.3 ± 1.5 (8)		12.83 (8)	13.1		18.1 ± 1.2
31	Sucrose	358	302	10.8	34.1	10.8	39.4	— 59.7 (4)	— 16.1 ± 1.5 (5)	7.0	12.58 (5)	13.9	6.0	16.3 ± 3.2
32	+ 1% sulfasuxidine	350	302	11.9	49.7	25.2	37.6	— 58.3 (5)	— 13.1 ± 1.5 (5)		12.90 (5)	13.2	7.3	14.7 ± 3.5
33	+ 0.1% DL-threonine	351	293	11.0	35.1	10.3	34.5	— 61.5 (5)	— 9.6 ± 1.4 (5)		10.15 (5)	15.2	8.2	7.4 ± 1.0
	Experiment VII — 0.1% EAA + 0.1% NEAA (Wistar-strain rats)												
34	Sucrose	352	285	11.9	37.5	12.8	40.6	— 66.5 (11)	— 15.9 ± 1.1 (11)		13.84 (11)	12.4		20.0 ± 1.4
35	Corn dextrin	349	287	11.9	43.0	15.1	35.2	— 64.5 (11)	— 7.3 ± 0.9 (11)		11.01 (11)	15.0		9.3 ± 1.1
36	Corn dextrin [1]	356	282	11.6	41.7	16.9	37.7	— 67.4 (6)	— 12.8 ± 0.8 (6)		14.67 (6)	14.7		10.4 ± 2.3
37	Sucrose, ad libitum	353	301	13.2	41.0	14.2	38.5	— 66.0 (6)	— 11.7 ± 1.0 (6)		14.12 (6)	12.6	5.7	21.5 ± 2.0
38	Corn dextrin, ad libitum	355	307	13.9	49.9	18.1	36.6	— 64.6 (6)	— 4.8 ± 0.6 (6)		11.11 (6)	14.8	7.1	11.0 ± 1.8

[1] In experiment I the carbohydrate fed in the nitrogen-free period is indicated (groups 1a–7a). All other animals were depleted on diets containing sucrose except group 16 (wheat starch) and group 36 (corn dextrin).

[2] Numbers in parentheses indicate number of animals per group.

[3] Difference between means required for significance, P = 0.05%; this test difference reproduces the results of Duncan's method of analysis ('54).

[4] Wet basis.

[5] Average weight = 361 gm.

[6] EAA, essential amino acids; NEAA, non-essential amino acids.

[7] Insufficient number of determinations.

was 370 to 379 gm. Comparisons of nitrogen balances of animals receiving these rations should be made only between groups of animals of comparable weights.

The fecal nitrogen excreted by the animals depended on the carbohydrate fed and on its prior treatment. For example, the daily fecal nitrogen of group 1b (sucrose) was 11.7, of 3b (raw cornstarch) 13.0, and of 4b (cooked cornstarch) 20.1 mg. The differences in fecal nitrogen apparently were not due to adsorption of dietary nitrogen since fecal nitrogen values for the nitrogen-free periods were about the same as those for the periods in which amino acids were fed(see groups 1a and 1b, etc.). These results suggest that caution should be exercised in interpreting results in studies of the digestibility of proteins in which relatively large amounts of protein-containing food in the diet change the proportions of carbohydrate from those of the ration used for the determination of "endogenous" fecal nitrogen.

Grams of fat, protein and glycogen per liver, while not included in this report, are easily estimated from table 1. As previously reported (Marshall and Womack, '54) the grams of protein ($N \times 6.25$) per liver were higher in fatty livers than in those in which the fat was more nearly normal. For example, groups 1b and 4b, with 16.5 and 7.1% fat had 1.64 and 1.49 gm of protein, respectively.

The glycogen in the livers varied from 4.2 (stock rats) to 10.0% (group 13, table 1). When glycogen determinations were made, the animals were allowed to eat ad libitum for 24 hours after the end of the period during which urine and feces were collected so that liver glycogen values would not be influenced by lack of food for varying lengths of time. The variables introduced by this procedure may have influenced the glycogen content of the liver. For this reason, differences in glycogen formation from the different carbohydrates cannot be accurately assessed. Moreover, due to variations in liver weight, errors may be introduced into conclusions based on percentages of liver glycogen. In groups 18, 19 and 20, for example, liver glycogen values were 6.0,

5.8 and 6.2%. However, these values indicate 0.79, 0.55 and 0.83 gm of glycogen per liver. On the other hand, groups 16 and 17 had 8.9 and 5.5% of glycogen which represent 0.96 and 0.86 gm of glycogen per liver.

SUMMARY

Liver fat was reduced and negative nitrogen balances were decreased in adult protein-depleted rats fed extra threonine, corn, rice or wheat starch, or corn dextrin, when compared with the levels found for animals fed diets containing sucrose and low levels of amino acids. The results were not influenced by the type of carbohydrate fed during protein depletion. Substitution of potato starch or glucose for sucrose reduced liver fat but did not improve nitrogen balances. The addition of niacin, methionine, cellu flour or sulfasuxidine, or substitution of fructose for sucrose did not change liver fat values or nitrogen balances from those obtained with sucrose. Doubling the essential amino acid intake of animals receiving sucrose, glucose or fructose reduced liver fat to normal or near normal values.

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LITERATURE CITED

DUNCAN, D. B. 1954 Multiple range and multiple F tests. Technical report No. 9, Virginia Agricultural Experiment Station.

HARPER, A. E., W. J. MONSON, D. A. BENTON AND C. A. ELVEHJEM 1953a The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. J. Nutrition, *50:* 383–393.

HARPER, A. E., W. J. MONSON, D. A. ARATA, D. A. BENTON AND C. A. ELVEHJEM 1953b Influence of various carbohydrates on the utilization of low protein rations by the white rat. II. Comparison of several proteins and carbohydrates. Growth and liver fat. Ibid., *51:* 523–537.

JONES, J. H., AND C. FOSTER 1942 A Salt mixture for use with basal diets either low or high in phosphorus. Ibid., *24:* 245–256.

LITWACK, G., L. V. HANKES AND C A. ELVEHJEM 1952 Effect of factors other than choline on liver fat deposition. Proc. Soc. Exp. Biol. Med., *81:* 441–445.

MARSHALL, M. W., AND M. WOMACK 1954 Influence of carbohydrate, nitrogen source and prior state of nutrition on nitrogen balance and liver composition in the adult rat. J. Nutrition, *52:* 51–64.

MILLER, M., W. R. DRUCKER, J. E. OWENS, J. W. CRAIG AND H. WOODWARD, JR. 1952 Metabolism of intravenous fructose in normal and diabetic subjects. J. Clin. Invest., *31:* 115–125.

SEIFTER, S., S. DAYTON, B. NOVIC AND E. MUNTWYLER 1950 The estimation of glycogen with the anthrone reagent. Arch. Biochem., *25:* 191–200.

SHILS, M. E., I. FRIEDLAND AND W. B. STEWART 1954 Rapid development of portal fatty liver in rats consuming various plant materials. Proc. Soc. Exp. Biol. Med., *87:* 473–476.

SINGAL, S. A., S. J. HAZAN, V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1953 The production of fatty livers in rats on threonine- and lysine-deficient diets. J. Biol. Chem., *200:* 867–874.

SMITH, L. H., JR., R. H. ETTINGER, D. SELIGSON AND S. LIGHTCAP 1953 A comparison of the metabolism of fructose and glucose in hepatic disease and diabetes mellitus. J. Clin. Invest., *32:* 273–282.

WOMACK, M., M. W. MARSHALL AND A. B. PARKS 1953 Some factors affecting nitrogen balance in the adult rat. J. Nutrition, *51:* 117–130.

THE EFFECTS OF PYRIDOXINE DEFICIENCY ON SOME PHYSIOLOGICAL FACTORS OF IMPORTANCE IN RESISTANCE TO INFECTION

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The influence of nutritional deficiencies upon the susceptibility of animals to infectious disease has been studied and reported by numerous investigators. Animals maintained on diets deficient in various members of the vitamin B complex group have demonstrated a marked increase in susceptibility to certain bacterial and rickettsial infections, while increased resistance to certain viral infections has been reported in animals maintained on some vitamin-deficient diets. The basic mechanisms of this altered susceptibility to infection have not been adequately investigated. However, the ability of vitamin-deficient animals to produce circulating antibodies of various types has been rather extensively studied. The work in these fields has been recently reviewed elsewhere (Wertman, Smith, and O'Leary, '54).

Several observations on the effect of a niacin-tryptophan deficiency in the white rat were presented in an earlier paper (Wertman et al., '54). The non-specific physiological factors studied, which were believed to be of importance in resistance to infection, were: (1) complete blood count; (2) complement activity; (3) quantitative and qualitative cellular migration in inflammation; (4) capillary permeability; and (5) cellular composition of the bone marrow. A considerable decrease

of complement activity and a substantial reduction in cellular migration to an inflamed area were observed in the white rats maintained on a niacin-tryptophan deficient diet. Although a significant reduction in cellular migration occurred in the deficient animals, no alteration in capillary permeability could be measured by the Menkin dye accumulation technique (Menkin, '40).

The purpose of the investigation to be reported here was to perform similar studies in white rats maintained on a well-defined diet that was deficient in pyridoxine. Similar studies have been completed on rats fed diets deficient in folic acid, riboflavin and vitamin B_{12}. These results will be presented in future papers.

EXPERIMENTAL

Male weanling albino rats of the Sprague-Dawley strain, approximately 21 days old, were employed in this investigation. All animals were housed individually in wide-meshed screen bottom metal cages provided with glass water bottles and food dishes which were replenished daily. The animals were divided into a ad libitum control group, an inanition control group, and a pyridoxine-deficient group.

The basal diet was a modification of that employed by Wertman and Sarandria ('51). The ingredients used in the preparation of the basal diets were obtained from commercial sources [1] and were of the highest purity available. The basal diet had the following percentage composition: sucrose, 63.75; casein (vitamin free), 20; hydrogenated vegetable oil, 10; salt mixture number two, 4; corn oil, 2; choline chloride, 0.20; i-inositol, 0.03; para-amino-benzoic acid, 0.01; d-alpha-tocopherol acetate, 0.01; and 2-methyl-l, 4-naphthoquinone, 0.0001.

Each animal was carefully fed one vitamin pill daily. The pills prepared for the control animals contained the following vitamins in micrograms (Griffith and Farris, '49): niacin, 150; thiamine, 40; riboflavin, 60; pyridoxine, 50; calcium

[1] General Biochemicals Company, Inc., Chagrin Falls, Ohio. Nutritional Biochemicals Company, Cleveland, Ohio.

pantothenate, 150; folic acid, 1; and biotin, 1. Lactose was used as the binder in the preparation of the pills. Pyridoxine was omitted from the pills prepared for the vitamin-deficient animals.

All animals were fed the basal diet and control pills until weights of approximately 45 gm were attained. Thereafter, deficient animals received vitamin pills lacking pyridoxine. Ad libitum-control and deficient rats received the diet ad libitum while inanition-control animals were fed enough to maintain their weights equal to those of the deficient animals with which they were paired.

In addition to the vitamins supplied in the basal diet and supplementary pills, each animal was administered by mouth

TABLE 1

Distribution and initial and final mean weights of rats

GROUP	NUMBER OF RATS	MEAN WEIGHTS	
		Initial	Final
		gm	*gm*
Ad libitum controls	9	46.3	290.5
Inanition controls	13	47.5	130.1
Pyridoxine deficients	40	45.9	128.9

two drops of haliver oil [2] weekly which supplied 3000 U.S.P. units of vitamin A and 24 U.S.P. units of vitamin D. The animals were maintained on the basal diet and vitamin preparations for a period of 8 weeks. Initial and final mean weights for each group appear in table 1.

The day before the rats were to be sacrificed, blood samples for complete blood counts were obtained from all animals by tail bleeding. Standard hematological techniques were employed for these counts, the results of which are recorded in table 2. Following the tail bleeding, each rat was injected intraperitoneally with 10 ml of an inflammation-inciting fluid. This fluid was a sterile mixture of Locke's solution and double strength nutrient broth in the ratio of 85 to 15 parts on a volume basis.

[2] Abbott.

Twelve hours after the intraperitoneal injections, the rats were anesthetized with ether and exsanguinated by the cardiac puncture technique (Griffith and Farris, '49). The blood specimens so obtained were permitted to clot and the sera were collected, pooled, and utilized for the determination of complement activity as described in a previous paper (Wertman et al., '54). The results of these determinations are

TABLE 2

Cell counts and differential enumeration of peripheral blood in pyridoxine-deficient and control rats

		DIET		
		Ad libitum control (9)[1]	Inanition control (13)	Pyridoxine-deficient (40)
Total count				
R.B.C. × 1 × 10^4 cells/mm^3	Median	912	1,128	994
	M. D.[2]	9.8	11.1	15.8
	Range	816–1,005	917–1,281	836–1,165
W.B.C. cells/mm^3	Median	13,025	14,850	6,550
	M. D.	230	218	230
	Range	9,650–15,250	8,400–15,850	3,350–8,750
Differential count[3]				
		%	%	%
Neutrophiles Segmented	Median	9.5	6.5	21.0
	M. D.	1.0	1.4	2.2
	Range	4–15	3–8	16–33
Eosinophilic	Median	2.0	2.5	7.5
	M. D.	0.4	0.5	1.2
	Range	0–3	0–3	5–12
Lymphocytes Large and small	Median	88.0	89.5	74.0
	M. D.	0.9	1.5	2.0
	Range	79–94	82–100	54–86
Monocytes	Median	1.5	2.5	2.5
	M. D.	0.3	0.6	0.4
	Range	1–3	1–4	0–4

[1] Numbers of rats given in parentheses.

[2] Mean deviation.

[3] Band, basophile and blast cell enumeration were also conducted but did not exceed one per cent in any instance and are not included in the table.

recorded in table 3 and stated as the volume of a 1:6 dilution of pooled sera representing one exact unit of complement.

Immediately following the cardiac bleeding, the peritoneal exudates were collected from the anesthetized animals by washing the peritoneal cavity with heparinized Locke's solution (Wertman et al., '54). The exudates plus washings were centrifuged at low speed to remove all the cellular elements

TABLE 3

Complement activity of pyridoxine-deficient and control rats

GROUP	SERUM POOL[1]	COMPLEMENT ACTIVITY E.U.[2]
Ad libitum controls	1	0.06
	2	0.06
Inanition controls	1	0.09
	2	0.09
Pyridoxine deficients	1	0.12
	2	0.09
	3	0.15
	4	A.C.[3]
	5	0.09
	6	0.12
	7	0.09
	8	0.15

[1] Pooled sera of 5 rats for deficient and ad libitum control rats and pools of 6 for inanition control group.

[2] One exact unit.

[3] Anti-complementary.

which were then resuspended in 2 ml of Locke's solution for total and differential leucocyte counts. Standard techniques were employed for the total counts. The differential counts were performed on smear preparations of the cell suspensions which were stained by Wright's method. Two hundred cells were counted on each slide using the methods of Menkin ('40, '50) and Maximow and Bloom ('44) for classification. The results of the total and differential counts of the exudate cells obtained from the inflamed areas appear in table 4.

Samples of each cell-free exudate supernatant were concentrated 10-fold by the evaporation of water from dialysis bags as described in the previous paper (Wertman et al., '54) and pooled in groups of 5 specimens each. In an effort to determine the presence and activity of "leukotaxine" in the concentrated exudate supernatant, Menkin's intradermal dye-accumulation technique was employed (Menkin, '40). Two tenths milliliter of pooled exudate concentrate was injected intradermally into rabbits' shaved abdomens along with con-

TABLE 4

Leucocyte count and differential enumeration of inflammatory exudate in pyridoxine-deficient and control rats

EXUDATE CELLS		DIET		
		Ad libitum control (9)[1]	Inanition control (13)	Pyridoxine-deficient (40)
Total leucocytes cells/mm³	Median	16,050	10,875	11,325
	M. D.[2]	1,894	1,321	975
	Range	10,500–17,850	9,200–14,850	10,400–15,950
Granulocytes, %	Median	40.8	47.0	59.5
	M. D.	3.2	5.6	8.7
	Range	30.5–48.5	32.5–60.5	38.5–76.5
Cells/mm³	Median	6,602	5,699	6,639
	M. D.	996	1,067	926
	Range	3,461–8,099	3,591–7,425	4,336–8,953
Lymphocytes, %	Median	31.5	26.5	19.5
	M. D.	5.3	4.2	5.8
	Range	24.5–49.0	16.5–37.0	8.5–38.5
Cells/mm³	Median	5,348	3,285	2,215
	M. D.	716	480	707
	Range	3,307–6,359	1,914–4,088	943–4,005
Monocytes, %	Median	22.3	24.5	22.5
	M. D.	4.1	5.1	4.6
	Range	18.0–32.5	12.0–36.0	11.0–32.5
Cells/mm³	Median	3,628	2,724	2,470
	M. D.	977	681	531
	Range	2,326–5,801	1,104–4,158	1,160–4,392

[1] Number of rats given in parentheses.

[2] Mean deviation.

TABLE 5

Cellular composition of the bone marrow in pyridoxine-deficient and control rats

BONE MARROW CELLS		DIET		
		Ad libitum control	Inanition control	Pyridoxine-deficient
		%	%	%
Nucleated red cells	Median	44.2	43.4	41.7
	M. D.[1]	4.7	5.3	5.6
	Range	39.3–54.3	32.0–54.3	30.0–50.0
Total granulocytes	Median	35.7	42.4	47.7
	M. D.	5.6	5.8	5.7
	Range	24.0–44.3	32.7–52.3	31.0–61.3
Metamyelocytes and segmenters	Median	24.8	29.0	35.0
	M. D.	4.6	4.5	5.0
	Range	18.0–32.0	22.0–39.0	21.0–42.7
Myelocytes and premyelocytes	Median	6.0	7.0	8.3
	M. D.	2.1	2.1	2.6
	Range	4.0–10.3	4.0–11.3	3.0–20.0
Lymphocytes	Median	11.5	6.5	3.0
	M. D.	3.1	2.1	1.6
	Range	7.7–16.0	4.0–12.3	1.0–9.0
Blast cells	Median	2.2	2.0	2.0
	M. D.	0.9	1.2	1.2
	Range	0.7–4.0	0.0–3.0	0.0–5.7
Monocytes	Median	1.2	1.1	1.0
	M. D.	0.6	0.6	0.6
	Range	0.0–2.7	0.0–3.0	0.0–3.7
Eosinophiles	Median	4.0	3.8	4.3
	M. D.	1.3	0.8	1.4
	Range	2.3–7.0	2.0–5.3	1.0–8.3
Plasma cells	Median	0.9	1.1	1.3
	M. D.	1.4	0.6	0.9
	Range	0.0–2.0	0.3–3.0	0.0–3.0
Mast cells	Median	0.9	1.8	1.8
	M. D.	0.9	0.7	0.6
	Range	0.0–3.7	0.7–3.0	0.0–5.3
Unclassified	Median	1.2	1.5	1.7
	M. D.	0.6	0.9	0.8
	Range	0.0–2.3	0.0–3.7	0.0–3.3

[1] Mean deviation.

trol injections of saline, heparinized Locke's solution and the inciting fluid. Ten milliliters of sterile 1% trypan blue were injected into each rabbit's marginal ear vein immediately after the intradermal injections were completed. The sites of the intradermal injections were observed and measurements of the areas of dye accumulation made after a period of 30 minutes.

Following the cardiac bleedings and removal of peritoneal exudates, bone marrow specimens were taken from each rat by cutting through the proximal end of the tibia and removing a portion of the marrow so exposed. The marrow was mixed with a drop of normal rabbit serum and smeared across a slide. The resultant films were stained with Wright's stain and differential counts made. Three hundred cells were observed on each slide using a modification of the method of Endicott and Ott ('45) to classify them. The results of these counts appear in table 5.

RESULTS AND DISCUSSION

The results of this investigation indicated no alteration of the peripheral erythrocyte count due to either inanition or vitamin deficiency. However, a characteristic microcytosis was observed in the blood of the rats maintained on a pyridoxine-deficient diet. The lack of pyridoxine, and not the concomitant inanition, produced a leucopenia in the rat. Pyridoxine deficiency per se, and not concomitant inanition, resulted in a percentage increase in polymorphonuclear leucocytes and an eosinophilia.

The titration of rat sera indicated that inanition alone could bring about a slight reduction in complement activity. However, the sera of pyridoxine-deficient rats were found to have a greater reduction of complement activity, although the reduction of activity was not as great as observed in a previous study with niacin-tryptophan-deficient animals (Wertman et al., '54). The sera of the two groups of ad libitum-control rats contained one exact unit in 0.06 ml of a 1:6 dilution. The two pools of sera formed from the rats on an inanition

diet contained one exact unit of 0.09 ml of a 1:6 dilution. The vitamin-deficient sera tested as follows: three groups of pooled sera contained one exact unit of 0.09 ml of 1:6 dilution, two groups contained one exact unit 0.12 ml of a 1:6 dilution and two groups contained one exact unit in 0.15 ml of a 1:6 dilution.

The total leucocyte counts of the inflammatory exudates removed from the inanition and pyridoxine-deficient groups demonstrated a reduction in number over the ad libitum controls. A median count of approximately 15,750 cells/mm^3 was recorded for the ad libitum animals, while median counts of 10,900 and 11,500 cells/mm^3 were noted for the inanition control and vitamin-deficient groups respectively. The fact that both vitamin deficiency and inanition had approximately the same level of response suggests that the decrease in total leucocytes might be due to the reduced nutritional intake rather than pyridoxine deficiency.

Differential counts of the exudates demonstrated relative granulocytosis and lymphopenia in the inanition animals as compared with the ad libitum controls. The median percentage of granulocytes was 47.0 for inanition animals and 40.8 for the ad libitum animals, while the median percentage of lymphocytes was 26.5 for the inanition animals and 31.5 for the ad libitum controls. In the exudates from the pyridoxine-deficient animals, relative granulocytosis and lymphopenia were observed, both of which were considerably more marked than the corresponding changes found in the inanition controls. The median percentage of granulocytes in the deficient group was 59.9 and that of lymphocytes in the same group was 19.5. Mononuclear cells showed little relative change in any group.

Experiments were performed to determine whether or not "leukotaxine" activity was present in those animals which demonstrated a reduction in cellular migration to an inflamed area. No alteration in capillary permeability could be detected by the Menkin dye-accumulation technique. In every instance, the cell free exudate from all groups of animals

produced the same type and degree of reaction in the skin of the rabbit after the injection of dye. It was evident that the reduction in cellular response was not due to the inability of the rats to produce the material and reaction described by Menkin.

The bone marrow of the inanition animals demonstrated a moderate increase in granulocytes and decrease in lymphocytes as compared with the ad libitum controls. The median percentage of granulocytes was 42.4 for the inanition animals and 35.7 for the ad libitum animals, while the median percentage of lymphocytes was 6.5 for the inanition animals and 11.5 for the ad libitum controls. The marrow of the pyridoxine-deficient animals showed granulocytosis and lymphopenia, both of which were considerably more marked than the corresponding changes observed in the inanition controls. The median percentage of granulocytes in the deficient group was 47.7 and that of lymphocytes in the same group was 3.0.

SUMMARY

Male white rats were maintained on a well-defined diet deficient in pyridoxine, and various physiological factors of resistance to infection were studied. Adequate inanition- and ad libitum-control animals were included. The following physiological factors were studied: (1) cellular composition of the peripheral blood; (2) complement activity; (3) cellular migration in inflammation; (4) cellular composition of the exudate in inflammation; (5) "leukotaxine" activity, and (6) cellular composition of bone marrow.

The following observations were made:

(1) No change was noted in the total erythrocyte count in the peripheral blood of the vitamin-deficient, inanition-, and ad libitum-control animals. A microcytosis was observed in the blood of pyridoxine-deficient rats. The lack of pyridoxine, and not the concomitant inanition, produced a leucopenia in the deficient rats.

(2) Differential cell counts of the blood of pyridoxine-deficient rats showed a percentage increase in polymorphonuclear leucocytes and a percentage decrease in lymphocytes.

(3) Complement activity in the sera of pyridoxine deficient rats was less than in either inanition or ad libitum controls.

(4) Cellular migration to an inflamed area was reduced in pyridoxine-deficient and inanition-control animals as compared with ad libitum controls.

(5) Relative granulocytosis and lymphopenia were observed in the exudates of inanition controls as compared with ad libitum controls. Exudates of pyridoxine-deficient rats demonstrated relative granulocytosis and lymphopenia, both of which were more marked than corresponding changes in inanition controls.

(6) No alteration in capillary permeability, as measured by the Menkin dye-accumulation technique, was noted in any group.

(7) Relative granulocytosis and lymphopenia were observed in the bone marrow of inanition controls as compared with ad libitum controls. Bone marrow of pyridoxine-deficient rats demonstrated relative granulocytosis and lymphopenia both of which were more marked than corresponding changes in inanition controls.

LITERATURE CITED

ENDICOTT, K. M., AND M. OTT 1945 The normal myelogram in albino rats. Anat. Rec., *92:* 61.

GRIFFITH, J. Q., AND E. FARRIS 1949 The Rat in Laboratory Investigation. J. P. Lippincott Co., Philadelphia, Pa., 2nd Ed.

MAXIMOW, A. A., AND W. A. BLOOM 1944 Textbook of Histology. W. B. Saunders Co., Philadelphia, Pa., 4th Ed.

MENKIN, V. 1940 Dynamics of Inflammation. Macmillan Co., New York, N. Y.

———— 1950 Newer Concepts of Inflammation. Charles C Thomas Co., Springfield, Ill.

WERTMAN, K., AND J. L. SARANDRIA 1951 Complement-fixing murine typhus antibodies in vitamin deficiency states. II. Pyridoxine and nicotinic acid deficiencies. Proc. Soc. Exp. Biol. Med., *78:* 332.

WERTMAN, K., L. W. SMITH AND W. M. O'LEARY 1954 The effects of vitamin deficiencies on some physiological factors of importance in resistance to infection. I. Niacin-tryptophane deficiency. J. Immun., *72:* 196.

DENTAL CARIES IN THE ALBINO RAT IN RELATION TO THE CHEMICAL COMPOSITION OF THE TEETH AND OF THE DIET

I. EFFECT OF PRENATAL AND POSTNATAL FEEDING OF HIGH PROTEIN, HIGH FAT AND HIGH CARBOHYDRATE DIETS

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In previous experiments (Haldi and Wynn, '52) we found that a diet containing 64% of sucrose is more cariogenic when fed to albino rats of the Wistar strain in our colony than a high-protein or high-fat diet containing approximately 20% of sugar. Hartles ('51) had previously reported that the enamel and dentin of the incisors of rats maintained on a 67% sucrose diet contained significantly greater amounts of calcium and phosphorus than the same tissues of animals that had been fed a stock diet. On the high-sugar diet the Ca/P ratios of the enamel and dentin were significantly lower than on the stock diet.

These observations suggested the possibility that a preponderance of one of the major foodstuffs in the diet might conceivably affect the mineral composition of the molar teeth and thereby make them more or less resistant to caries. The experiments reported herein were undertaken to investigate this possibility.

Two different ways by which the diets could conceivably affect the composition of the teeth were taken into consideration: (1) postnatally, by systemic mechanisms or by intraoral

ionic exchanges after tooth eruption (Volker and Sognnaes, '40; Sognnaes and Volker, '41; Armstrong, '42; Sognnaes and Shaw, '52; Sognnaes, Shaw, Bogoroch and Sweeney, '52; Hansard and Plumlee, '54) and (2) by some prenatal influence (Sognnaes, '48, '50).

EXPERIMENTAL

The postnatal influence of diet was studied by placing weanling rats on a high-sucrose, a high-protein or a high-fat diet (table 1) for 150 days and making chemical analyses of the teeth at the end of this time. These animals were born of parents that had been raised on a commercial stock diet.[1] They were selected in groups of three of the same sex and from the same litter and will be referred to as the first-generation animals.

In order to study the prenatal influence of the synthetic diets it was necessary to (1) obtain second-generation animals, i.e., offspring of mothers that had been fed these diets from the time they were weaned, up to and including the periods of pregnancy and lactation; (2) feed these animals the same high-sucrose, high-protein and high-fat diets that had been fed their mothers; and (3) compare the composition of the teeth of the second-generation animals with that of the teeth of rats of the first generation.

If the animals used in the first part of the experiment (which we have designated as the first-generation animals) had been bred to provide offspring for the second part of the experiment (the second-generation animals), the variables of pregnancy and lactation would have been introduced and these conceivably might have affected the composition of the teeth of the first-generation animals. This dilemma was resolved by raising another group of animals under identical conditions as the first-generation animals solely to obtain offspring for the second part of the experiment. It is these offspring that are referred to as second-generation animals. They, also, were fed the experimental diets for 150 days from

[1] Purina Laboratory Chow.

the time of weaning. There were 10 animals on each of the three diets in the first and in the second generation. All were allowed to eat ad libitum.

At the conclusion of the experiment the animals of both generations were sacrificed and the teeth removed for chemi-

TABLE 1

Composition of the high-sucrose, high-protein and high-fat diets

CONSTITUENTS	DIET 1	DIET 2	DIET 3
	%	%	%
Sucrose	64	22	24
Casein	20	62	25
Fat	8	8	43
Yeast and liver extract	4	4	4
Salt mixture	4	4	4

FROM SALT MIXTURE	*gm per 100 gm diet*	VITAMINS [1]	*mg per 100 gm diet*
Calcium	0.60	Choline	300.0
Phosphorus	0.38	Linoleic acid	50.0
Magnesium	0.053	α-Tocopherol	30.0
Potassium	0.72	Inositol	13.0
Sodium	0.40	Niacin	3.2
		Pantothenic acid	2.5
	mg per 100 gm diet	Riboflavin	2.4
Iron	48.0	Thiamine	2.4
Sulfur	31.0	Pyridoxine	1.0
Manganese	7.8	Folic acid	0.1
Copper	1.0	Carotene	0.3
Zinc	0.4	Biotin	0.012
Iodide	0.15	A concentrate (I.U. per 100 gm)	1000.0
Fluoride	0.01	Viosterol (U.S.P. units per 100 gm)	300.0
Aluminum	0.016		

[1] From yeast and liver extract and additional supplements. To make the diets more palatable 1.6 ppm of butter flavor was added.

cal analyses. In order to obtain sufficient tooth substance for the complete analyses it was found necessary to pool all the molar teeth of each individual rat. Each analytical datum obtained from both the first- and second-generation animals therefore represented an average value for 12 molar teeth.

After extraction the teeth were cleaned and dried to constant weight in a vacuum desiccator over P_2O_5. The pooled teeth of each rat were then ground to pass through a 60-mesh screen and re-dried to constant weight. Aliquots were taken for analysis for nitrogen, calcium, phosphorus, magnesium, carbon dioxide and fluoride. The analytical procedures were as follows: nitrogen by the micro-kjeldahl method; calcium, Sobel, Rochenmacher and Kramer ('44); phosphorus, Fiske and Subbarrow ('25); carbon dioxide, Van Slyke and Folch ('40) as modified by Sobel, Rochenmacher and Kramer ('44); fluoride, Methods of Analysis, A. O. A. C. ('50), with titration as described by Williams ('46); magnesium, Young and Gill ('51). The presence of trace elements was determined by spectrographic analysis.

RESULTS AND DISCUSSION

The composition of the teeth of the first- and second-generation animals is given in table 2. In addition it should be stated that the teeth of the animals of both generations on the three diets were found to contain traces of Si, Ti, B, As, Sb, Na, Cu, Al, K, Sn, Ag, Sr, Pb, Zn and Fe.

Postnatal influence of diet. The animals of the first generation, as stated previously, were placed on the high-sucrose, high-protein or high-fat diets at weaning. Analysis of the teeth of these animals should therefore reveal any effect that might result from postnatal feeding of the different diets.

Inspection of table 2 shows that the calcium, phosphorus, magnesium, fluoride and CO_2 content of the teeth were the same on the three different diets. The nitrogen was slightly higher on the sucrose diet. Postnatal feeding of a diet with a preponderance of any one of the major foodstuffs therefore had no effect on the mineral content of the molar teeth. It should be noted that the larger amount of casein in the high-protein diet resulted in a higher phosphorus content of this diet, namely, 0.84 as compared with 0.51% in the other two diets. This larger amount of phosphorus reduced the Ca/P ratio to 0.6:1 as compared with a ratio of 1:1 in the high-

TABLE 2

Chemical analyses of the molar teeth of albino rats maintained on high-sucrose, high-protein or high-fat diets

DIET	NO. OF ANIMALS	N	Mg	CO_2	F	Ca	P	Ca/P
		%	%	%	%	%	%	
				First generation				
High sucrose	10	2.40 ± 0.08 [1]	0.29 ± 0.05	3.46 ± 0.18	0.012 ± 0.004	28.6 ± 0.42	13.9 ± 0.31	2.05 ± 0.05
High protein	10	2.33 ± 0.04	0.29 ± 0.05	3.51 ± 0.09	0.009 ± 0.003	28.7 ± 0.54	14.0 ± 0.30	2.06 ± 0.07
High fat	10	2.35 ± 0.09	0.29 ± 0.04	3.49 ± 0.14	0.011 ± 0.004	28.5 ± 0.81	13.9 ± 0.52	2.05 ± 0.08
				Second generation				
High sucrose	10	2.51 ± 0.08	0.29 ± 0.03	3.36 ± 0.12	0.011 ± 0.005	28.4 ± 0.35	13.9 ± 0.43	2.05 ± 0.07
High protein	10	2.40 ± 0.07	0.28 ± 0.09	3.37 ± 0.12	0.010 ± 0.007	28.0 ± 0.61	13.7 ± 0.49	2.05 ± 0.09
High fat	10	2.45 ± 0.09	0.27 ± 0.03	3.38 ± 0.15	0.012 ± 0.004	27.8 ± 1.00	13.6 ± 0.30	2.04 ± 0.06

[1] The ± values are the standard deviations (σ).

sucrose and high-fat diets. The ratios were determined from actual analyses of the calcium and phosphorus of the complete diet. These differences in the phosphorus content and Ca/P ratio in addition to the difference in the general character of the diet produced no demonstrable change in the mineral composition of the teeth.

Prenatal influence of diet. The second-generation, unlike the first-generation animals, came under the influence of the high-sucrose, high-protein and high-fat diets prenatally and during the period of lactation. The preponderance of any one of the major foodstuffs in the diet fed the mother during the formative and developmental stages of tooth structure had no effect, as shown in table 2, on the mineral composition of the teeth.

It is of interest to compare the composition of the diets fed the parents of the first- and of the second-generation animals. The latter were fed the high-sucrose, high-protein and high-fat diets, the composition of which is given in table 1; the former, the stock diet consisting solely of Purina Laboratory Chow. Our analysis of the stock diet gave the following values: calcium, 1.18%; phosphorus, 0.90%; magnesium, 0.19%; fluoride, 55 p.p.m. These values, except for fluoride, were practically identical with those reported by the manufacturers. The fluoride content according to our analysis was about twice the amount given in the factory report.

Notwithstanding the difference in the mineral content of the synthetic and stock diets which were fed the mothers of the first and second generations during pregnancy and lactation, it will be noted, by comparing the data on the first- and second-generation animals in table 2, that there was no difference in the composition of the teeth of the first- and second-generation animals except for the nitrogen and CO_2 content. There was slightly less nitrogen and slightly more CO_2 in the teeth of the first-generation animals with a high degree of probability that these differences were not chance variations. The critical ratios of the differences in nitrogen between the two generations on the high-sucrose, high-protein

and high-fat diets were 3.1, 2.8 and 2.5 respectively, and in the case of the CO_2 content 3.0, 3.1 and 2.2. It remains for future investigation to determine whether these small differences are of biological significance.

On the assumption that the synthetic and stock diets provided biologically adequate amounts of calcium, phosphorus, magnesium and fluoride, the identity of the mineral content of the teeth of the first- and second-generation animals in spite of the pronounced differences in the mineral composition of the diets fed the mothers during pregnancy and lactation, suggests the following hypothesis: genetically determined mechanisms operate to deposit in the teeth definite and characteristic quantities of these minerals if they are available within the organism at certain minimum levels; these mechanisms will not deposit greater quantities of the minerals even though they are available in larger amounts. Further experiments are now in progress in our laboratories to test this hypothesis by making drastic changes in the mineral content of the diet.

One of the purposes of this investigation was to determine whether the greater amount of caries produced by the high-sucrose diet as reported previously (Haldi and Wynn, '52) might be related to an effect of this diet on the mineral composition of the teeth. The experiments reported in this paper show that this was not the case.

Hartles ('51) found that the enamel and dentin of the incisors of rats fed a 67% sucrose diet contained significantly larger amounts of calcium and phosphorus than the same tissues of animals maintained on a stock diet. On the basis of these observations he proposed the hypothesis that a diet high in sucrose stimulates the processes of mineralization by maintaining a maximum level of glycogen in the developing tooth. Our experiments do not support this hypothesis inasmuch as the calcium and phosphorus content of the molar teeth were not affected prenatally or postnatally by a high-sucrose diet.

SUMMARY AND CONCLUSIONS

The postnatal effects of a high-sucrose, high-protein or high-fat diet on the mineral composition of the teeth of albino rats were studied by feeding these diets for a period of 150 days from weaning. The mothers of these animals had been fed only a stock diet.

The prenatal effects were studied by feeding these diets to animals whose mothers had been raised on these diets and fed the same diets during pregnancy and lactation.

The preponderance of any one of the major foodstuffs in the diet had neither a prenatal nor postnatal influence on the mineral composition of the teeth.

The greater cariogenicity of the high-sucrose as compared with the high-fat and high-protein diets which had been demonstrated previously, can not therefore be ascribed to any changes induced in the mineral composition of the teeth by a preponderance of sucrose in the diet

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LITERATURE CITED

ARMSTRONG, W. D. 1942 Biochemical and nutritional studies in relation to the teeth. Ann. Rev. Biochem., *11:* 441.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Methods of Analysis, 7th Ed. Washington, D. C.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

HALDI, J., AND W. WYNN 1952 Cariogenicity of sugar-containing diets. J. Nutrition, *46:* 425.

HANSARD, S. L., AND M. P. PLUMLEE 1954 Effects of dietary calcium and phosphorus levels upon the physiological behavior of calcium and phosphorus in the rat. Ibid., *54:* 17.

HARTLES, R. L. 1951 The effect of a high-sucrose diet on the calcium and phosphorus content of the enamel and dentine of rat incisors. Biochem. J., *48:* 245.

SOBEL, A. E., M. ROCKENMACHER AND B. KRAMER 1944 Microestimation of the inorganic constituents of bone. J. Biol. Chem., *152:* 255.

SOGNNAES, R. F. 1948 Caries-conducive effect of a purified diet when fed to rodents during tooth development. J. Am. Dent. Assn., *37:* 676.

———— 1950 Is the susceptibility to dental caries influenced by factors operating during the period of tooth development. J. Calif. State Dent. Assn., *26:* 37. (Supplementary issue.)

SOGNNAES, R. F., AND J. H. SHAW 1952 Salivary and pulpal contributions to the radiophosphorus uptake in enamel and dentin. J. Am. Dent. Assn., *44:* 489.

SOGNNAES, R. F., J. H. SHAW, R. BOGOROCH AND E. SWEENEY 1952 Preliminary studies on the diffusion of P^{32}, I^{131} and H^{3} through the teeth in Rhesus monkeys. J. Dent. Res., *31:* 483.

SOGNNAES, R. F., AND J. F. VOLKER 1941 Studies on the distribution of radioactive phosphorus in the tooth enamel of experimental animals. Am. J. Physiol., *133:* 112.

VAN SLYKE, D. D., AND J. FOLCH 1940 Manometric carbon determination. J. Biol. Chem., *136:* 509.

VOLKER, J. F., AND R. F. SOGNNAES 1940 A study of the phosphorus metabolism in the dental tissues of the cat by the use of radioactive phosphorus. J. Dent. Res., *19:* 292.

WILLIAMS, H. A. 1946 The titration of microgram quantities of fluorides. Analyst, *71:* 175.

YOUNG, H. Y., AND R. F. GILL 1951 Determination of magnesium in plant tissue with thiazole yellow. Anal. Chem., *23:* 751.

THE PERCENTAGE OF PROTEIN IN CORN AND ITS NUTRITIONAL PROPERTIES[1]

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The average percentage of protein in corn is less than 10 but this amount can be approximately doubled by suitable cultural practices. However, a considerable part of the protein of corn is zein, of low biological value, and this could mean that, at least for monogastric animals, it is of comparatively little importance whether the percentage of protein is high or low. According to Mitchell, Hamilton and Beadles ('52), as the percentage of protein in corn increases, the percentage of zein in the protein increases also and the biological value of the protein decreases. Sauberlich, Chang and Salmon ('53) reported that as the percentage of protein in corn increased the percentage of all the amino acids increased also but the percentages of arginine, glycine, lysine and tryptophan in the protein declined. Eggert, Brinegar and Anderson ('53) used weanling pigs as experimental animals and concluded that the protein in low-protein corn is superior to the protein in high-protein corn, but high-protein corn is superior to low-protein corn. Sauberlich, Chang and Salmon ('53) compared the nutritional value of samples of corn that contained 7.8 and 11.4% of protein. When their rations contained the same percentages of corn, high-protein corn was superior to low-protein corn. When the rations were supplemented with

[1] Contribution from the Missouri Agricultural Experiment Station, Journal series No. 1532. Approved by the director.

methionine, lysine and tryptophan the superiority of the high-protein corn was greatly accentuated. Dobbins, Krider, Hamilton, Earley and Terrill ('50) used rats and pigs to compare the nutritional properties of three corn samples with different percentages of protein. Each ration contained 23.5% of a protein supplement, and the proportion of corn in each ration was so varied that the percentage of total protein would be approximately 15.4. The animals in the three groups all grew at about the same rate, and if there were differences in the biological value of the protein in the three types of corn, the fact was not revealed. In another study each ration contained the same amount of corn, 74.5%, and the amount of protein supplement was reduced as the percentage of protein in the corn increased so that the total amount of protein in each ration was 15%. The rate of gain by the pigs on the ration that contained high-protein corn was distinctly under the rate by pigs on the other rations. Ross, Garrigus, Hamilton and Earley ('54) reported that high-protein corn is more useful for lambs than is low-protein corn.

Hogan ('17) supplied rats with a diet that contained a concentrate (33%) of corn protein and concluded that tryptophan is the first limiting factor in corn proteins and that lysine is the second. Mitchell and Smuts ('32) concluded that lysine and not tryptophan is the first limiting factor. It may be that there are definite differences between strains of corn, and that tryptophan was the first limiting amino acid in the strain Hogan used. However, even if lysine were the first limiting factor as determined by modern techniques, it seems plausible that Hogan's diet was seriously deficient in nicotinic acid, and the rats responded as they did because tryptophan is a precursor of that vitamin. In the experience of Sauberlich, Chang and Salmon ('53) there was no increase in the gains in weight when methionine and lysine were added to either a high- or a low-protein corn ration, which means lysine was not the first limiting factor in their samples of corn. When methionine and tryptophan were added to the high-protein corn ration the rats gained 50% more than did those on the

basal diet. This observation would indicate that tryptophan was the first limiting factor.

The object of this investigation was to determine in more detail what are the differences in nutritional properties of high- and of low-protein corn.

EXPERIMENTAL

The high-protein corn contained 16.1% of protein (N × 6.25), was white, and was grown in Illinois.[2] The low-protein corn, U.S. 13, contained 7.3% of protein, was yellow, and was grown in central Missouri. The experimental animals were albino rats. Wistar Institute stock was used in the investigations on high-protein corn, but was unavailable for the studies on low-protein corn and was replaced with the Sprague-Dawley strain. Six males approximately 21 days of age and weighing from 30 to 40 gm were placed on each ration. They were kept in individual cages, equipped with raised screen bottoms and special feeding devices, which permitted accurate measurements of food consumption. Food and water were supplied ad libitum. The rats were first fasted for 24 hours, though water was available, in an effort to reduce variability in the weight of the intestinal contents. Six control animals were subjected to the same procedure, but at the end of the 24-hour period they were slaughtered and the contents of the intestinal tract were weighed. It was assumed that the intestinal contents were the same percentage of the live weight in the experimental as in the control animals, and the initial empty weight of each experimental animal was calculated. At the end of 28 days the experimental animals were slaughtered, the contents of the alimentary tract removed and weighed in order to obtain the final empty weight. The difference between the initial and final empty weights is the gain in weight on which the calculations in the tables are based. When amino

[2] Supplied by L. F. Bauman, Department of Agronomy, University of Illinois, in July, 1950. Marked: 3437

acids were included in the diets they replaced an equal weight of corn. Lysine was supplied as the mono-hydrochloride but was calculated as the free base in the tables. The composition of the basal rations is shown in table 1.

TABLE 1

Composition of rations

	RATION						
	H[1]	XI	A[2]	K	W	Y	Z
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Corn (16.1% protein)	94.0	...	...	...	85.5	40.4	...
Corn (7.3% protein)	...	...	94.5	...	...	...	85.5
DL-Threonine[3]	0.5	...	...	...	...	...	...
DL-Methionine[4]	...	0.2	...	0.2	...	...	...
Casein[5]	...	17.7	...	8.6	...	...	...
Corn starch	...	76.7	...	85.8	...	45.1	...
Soybean oil meal	...	...	...	...	9.0	9.0	9.0
Salts (5)[6]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Calcium carbonate	1.0	0.9	1.0	0.9	1.0	1.0	1.0
Soybean oil	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid, mg	...	5.0	...	5.0	...	...	...
Total crude protein, %	15.3	15.3	7.0	7.9	17.4	10.4	10.4

Vitamins[7] per 100 gm of diet

Vitamin A	2000 I.U.	Pyridoxine hydrochloride	1.0 mg
Vitamin D	433 I.U.	Ca-pantothenate	3.0 mg
Vitamin E	2.5 mg	Choline chloride	100.0 mg
Vitamin K_3	2.5 mg	Biotin	20.0 μg
Thiamine hydrochloride	1.0 mg	Folic acid	500.0 μg
Riboflavin	1.0 mg	Vitamin B_{12}	2.0 μg

[1] Basal ration for high-protein corn diets.

[2] Basal ration for low-protein corn diets.

[3] Purchased from Distillation Products Industries, Rochester, N. Y.

[4] Courtesy of the Dow Chemical Co., Midland, Mich.

[5] Prepared by the method, with slight modification, of McCollum et al. ('22).

[6] Described by Richardson and Hogan ('46).

[7] A concentrate of vitamin A was purchased from Distillation Products Industries, Rochester, N. Y. The vitamin D was Delsterol, purchased from the E. I. du Pont de Nemours and Co., New Brunswick, N. J. Folic acid was supplied by the Lederle Laboratories, Pearl River, N. Y. All other vitamins were supplied by Merck and Co., Rahway, N. J.

RESULTS

Observations on high-protein corn

The results obtained with high-protein corn are summarized in table 2. The basal diet contained 304 mg % of lysine and 84 mg% of tryptophan. If all other essential amino acids were supplied at the levels required for optimum growth, diet II contained 30% of the recommended amount of lysine and 42%

TABLE 2

Amino acid deficiencies of high-protein corn

RATION	SUPPLEMENTS ADDED TO BASAL DIET	INTAKE		WEIGHT GAIN	WEIGHT GAIN / FOOD INTAKE	WEIGHT GAIN / PROTEIN INTAKE
		Food	Protein			
		gm	*gm*	*gm*	*gm/gm*	*gm/gm*
II	None	157	24.1	27.3	0.173	1.12
VI	N,[1] 5 mg	209	32.1	38.4	0.181	1.20
III	T, 0.2 gm	169	26.2	31.9	0.188	1.21
IV	L,[2] 1 gm	175	28.7	39.1	0.206	1.25
VII	N, 5 mg + T, 0.2 gm	209	32.4	39.7	0.186	1.22
VIII	N, 5 mg + L, 1 gm	251	41.1	63.4	0.250	1.54
V	L, 1 gm + T, 0.2 gm	299	49.5	94.9	0.318	1.92
IX	L, 1 gm + T, 0.2 gm + N, 5 mg	286	47.3	84.9	0.296	1.79
XI	Casein diet	317	48.6	117.4	0.370	2.41
Least significant difference	5% level	39.4	6.4	14.9	0.034	0.22
	1% level	52.5	8.6	19.9	0.046	0.29

Critical nutrients[3] per 100 gm of diet

	LYSINE	TRYPTOPHAN	NICOTINIC ACID	ZEIN	OTHER PROTEINS
	mg	*mg*	*mg*	*gm*	*gm*
In basal corn diet	304	84	2.1	5.6	9.6
In casein diet	1475	218	5.0	0	15.3

[1] N = nicotinic acid, L = L-lysine, T = DL-tryptophan.

[2] Courtesy of Dr. J. Waddell, E. I. du Pont de Nemours and Co., New Brunswick, New Jersey.

[3] Assay methods are described by Flynn et al. ('54).

of the recommended amount of tryptophan (Rose, Smith, Womack and Shane, '49). The difference in degree of deficiency is not large, but it seems reasonably certain lysine would be the first limiting amino acid and tryptophan would be the second, provided there were no complications with a deficiency of nicotinic acid.

Nicotinic acid. The data to be mentioned first were concerned with the improvement in nutritional value when nicotinic acid was added to diets that did not contain added lysine. Ration II was the basal diet, and ration VI was the basal diet with added nicotinic acid. Ration III contained added tryptophan, and this ration plus added nicotinic acid was ration VII. It will be noted that in both comparisons the mean gain in weight was distinctly higher when nicotinic acid was added to the diet, but the differences fell short of satisfactory statistical significance. However, if the combination of rations II and III is compared with the combination of rations VI and VII (added nicotinic acid), the difference in the amount of food consumed is significant at the 1% level, and the difference in weight gained is significant at the 5% level. It was unexpected that nicotinic acid would be more effective than tryptophan in accelerating gains in weight. There was no difference in the efficiency of utilization. A comparison of rations IV and VIII shows that when a ration includes lysine, the response to added nicotinic acid is significant at the 1% level by each criterion. Evidently 2.1 mg% of nicotinic acid is insufficient for the type of ration mentioned thus far. However, a comparison of rations V and IX shows that when a ration contains both lysine and tryptophan, there is no response to added nicotinic acid.

Tryptophan. (This amino acid was relatively ineffective in accelerating the rate of gain.) A comparison of rations II and III shows that when tryptophan alone was added to the basal diet there was a small increase in the rate of gain in weight. The statistical evidence of significance, however, is unsatisfactory as the calculations indicated that this result could be expected in only 70% of the attempts. However, we

feel that the failure to demonstrate statistical significance is due to the small number of animals. If the 6 animals on each diet are paired in order of their weights, every animal that received tryptophan is the heavier of the pair. It will also be recalled that the statistical evidence of significance of the difference of the means was weak when nicotinic acid was added to rations II and III and it was necessary to combine these groups in order to meet the usual criteria of significance. Furthermore, it is accepted that under some circumstances tryptophan is the precursor of nicotinic acid. When tryptophan was added to a ration that contained nicotinic acid, rations VI and VII, the increase was too slight to be given any consideration. The optimum amount of tryptophan in rations of the type described in table 2 is certainly more than 80 mg%, and is probably considerably less than 284 mg%. As would be expected, when tryptophan was added to a ration that contained added lysine (rations IV and VIII vs. rations V and IX), there were large and significant increases in the amount of food consumed, in the gains in weight, and in the efficiency of utilization.

Lysine. When lysine was added to the basal diet (compare rations II and IV) there was a definite increase in the weight gained, though the significance of the difference was under the 5% level. Again we feel that the failure to reach that level was due to the small number of animals. When lysine was added to a ration that already contained nicotinic acid (compare rations VI and VII), there was a large increase in the gain in weight which was significant at the 1% level. It will also be noted that significant differences were obtained in the amounts of food and protein consumed, and in the gain in weight per unit of food or of protein consumed. The optimum amount of lysine in a ration composed chiefly of high-protein corn is considerably more than 304 mg%, and considerably less than 1,304 mg%.

It was unexpected that the increases in weight gained on rations IV and VI would prove to be practically the same. One contained added nicotinic acid, the other contained added

lysine. One would conclude, though, that the rats on the lysine diet (ration IV) gained more nitrogen and water, and less fat, than did those on the nicotinic diet (ration VI).

Supplemented corn protein vs. casein. Of all of the corn rations No. V sustained the most rapid rate of gain in weight, 94.9 gm, but this was definitely inferior to the gain on the casein diet, 117.4 gm, on ration XI. The gains in weight per unit of food or of protein consumed were also superior on ration XI. Ration V contains a total of 1300 mg of lysine and 282 mg of tryptophan, but it did not sustain the maximum rate of gain. The casein ration, No. XI, is not markedly superior in these respects, and one would conclude that the corn ration is also mildly deficient in one of the other important essential amino acids. These two rations contain the same amount of total protein, but ration V contains 5.6% of zein, which presumably is of little nutritional value, unless supplemented. The proteins other than zein make up only 9.6% of the diet, as compared to 15.3% in the casein diet.

Observations on low-protein corn

The results of the study of low-protein corn are summarized in table 3. The basal diet contained 208 mg% of lysine and 54 mg% of tryptophan.

Nicotinic acid. The first point considered was whether or not the biological value of corn is improved by the addition of nicotinic acid, and the evidence is shown by comparison of the results on the following rations: A and E; B and F; C and G; and D and H. It will be observed that in no instance was there any increase in the gain in weight when nicotinic acid was included in the diet. It will be recalled that the high-protein corn diet was definitely improved by the addition of nicotinic acid. The required amount of nicotinic acid is not over 2.2 mg% in rations of this type.

Trytophan. The pattern of response when tryptophan was added to low-protein corn was about the same as on high-protein corn. The failure to obtain a response when the

diets did not contain added lysine is shown in the following comparisons: rations A and B; and rations E and F. When the rations contained added lysine there was a definite response to the further addition of tryptophan as shown in the following comparisons: rations C and D; and rations G and H. In each case the response was significant at the 1% level.

Lysine. The results with lysine were similar to those obtained in the high-protein corn series. The first comparisons

TABLE 3

Amino acid deficiencies of low-protein corn

RATION	SUPPLEMENTS ADDED TO BASAL DIET	INTAKE		WEIGHT GAIN	WEIGHT GAIN / FOOD INTAKE	WEIGHT GAIN / PROTEIN INTAKE
		Food	Protein			
		gm	*gm*	*gm*	*gm/gm*	*gm/gm*
A	None	148	10.3	19.2	0.129	1.86
E	N,[1] 5 mg	138	9.6	16.3	0.118	1.68
B	T, 0.2 gm	134	9.6	16.1	0.119	1.67
C	L,[2] 1 gm	145	10.8	22.8	0.155	2.07
F	N, 5 mg + T, 0.2 gm	141	10.1	16.2	0.113	1.59
G	N, 5 mg + L, 1 gm	155	11.6	23.1	0.150	2.00
D	L, 1 gm + T, 0.2 gm	173	13.3	36.7	0.212	2.74
H	L, 1 gm + T, 0.2 gm + N, 5 mg	154	11.8	36.2	0.235	3.07
K	Casein diet	283	22.5	91.3	0.323	4.06
Least significant difference	5% level	32.0	2.4	7.4	0.024	0.34
	1% level	42.8	3.2	9.8	0.032	0.45

Critical nutrients[3] per 100 gm of basal diet

	LYSINE	TRYPTOPHAN	NICOTINIC ACID	ZEIN	OTHER PROTEINS
	mg	*mg*	*mg*	*gm*	*gm*
In basal corn diet	208	54	2.2	1.7	5.3
In casein diet	715	106	5.0	0	8.0

[1] N = nicotinic acid; L = DL-lysine; T = DL-tryptophan.

[2] Courtesy of Dr. J. Waddell, E. I. du Pont de Nemours and Co., New Brunswick, New Jersey.

[3] Assay methods are described by Flynn et al. ('54).

considered were on the following rations: A and C, and E and G. The increase in gain in weight in each case did not quite reach significance at the 5% level. However, if a combination of rations A and E is compared with the combination of rations C and G (added lysine), the differences in gains in weight and in efficiency of utilization of the food are significant, at least at the 5% level. The differences in food intake were small and lacked significance. It seems quite certain that lysine was the first limiting amino acid in the low-protein corn we examined. The results when lysine was included in a diet that contained added tryptophan are shown in the following comparisons: rations B and D; and rations F and H. In each case, as would be predicted, the increases in food consumed, in gains in weight and efficiency of utilization, are significant at the 1% level.

As would be expected, the most rapid rate of gain was supported by rations D and H, which contain added lysine and tryptophan. The total amount of active lysine per 100 gm of diet was 706 mg and of tryptophan was 254 mg. The casein diet, which contained 8% of protein, supported about the same rate of growth as did ration V in table 2 which contained 15.2% of corn protein, and in addition was supplemented with lysine and tryptophan. The total amount of non-zein protein in these two rations is not markedly different. It seems quite certain that corn proteins are seriously deficient in some of the other essential amino acids. A comparison of tables 2 and 3 shows that the gains in weight are much the larger on high-protein corn, and this difference would be expected. High-protein corn contains 9.6% of "other proteins," while low-protein corn contains only 5.3%. High-protein corn contained about three times as much zein as low-protein corn but one can not be certain how useful it is as a source of nitrogen.

Adequacy of corn proteins in threonine

It had been suggested that the basal diet might be mildly deficient in threonine, and that possibility was investigated.

The results, summarized in table 4, indicate that threonine is not a limiting factor in high-protein corn. A comparison of rations I and II, also of X and IX, shows that the gain in weight was slightly lower when threonine was added to the diet. The possibility that low-protein corn may be deficient in threonine was not investigated.

TABLE 4

Corn protein as a source of threonine

RATION	SUPPLEMENTS ADDED TO BASAL DIET	INTAKE		WEIGHT GAIN	WEIGHT GAIN / FOOD INTAKE	WEIGHT GAIN / PROTEIN INTAKE
		Food	Protein			
		gm	*gm*	*gm*	*gm/gm*	*gm/gm*
I	None	163,8	25.0	29.0	0.18	1.15
II	Th,[1] 0.5 gm	156.8	24.1	27.3	0.17	1.12
X	L,[2] 1 gm + T, 0.2 gm	313.6	51.2	89.9	0.29	1.76
IX	L, 1 gm + T, 0.2 gm + Th, 0.5 gm	286.3	47.3	84.9	0.30	1.79

[1] Th = DL-threonine; L = DL-lysine; T = DL-tryptophan.

[2] Courtesy of Dr. J. Waddell, E. I. du Pont de Nemours and Co., New Brunswick, New Jersey.

A comparison of high-protein and low-protein corn

When corn is a major component of a ration it is customary to combine it with a protein supplement and a few examples of such combinations are summarized in table 5. Ration W contains 85.5% of high-protein corn and 9% of soybean oil meal with a total of 17.4% of crude protein in the ration. The average gain in weight was 91.1 gm. The efficiency of this protein mixture is considerably lower than that of casein, as is shown by a comparison with ration XI, table 2. Ration Z contains 85.5% of low-protein corn and 9% of soybean oil meal, with a total of 10.4% of crude protein. The animals made an average gain in weight of 79.9 gm, which is less than on high-protein corn. The rats on ration Y, which contains 40.4% of high protein corn, made an average gain in weight of 56.4 gm, the lowest of the three groups. Rations Y

TABLE 5

A combination of the proteins of soybean oil meal and of corn. A comparison of high-protein and low-protein corn

RATION	TYPE AND AMOUNT OF CORN IN RATION	INTAKE		WEIGHT GAIN	WEIGHT GAIN / FOOD INTAKE	WEIGHT GAIN / PROT. INTAKE	PROT. GAIN / PROT. INTAKE	CALORIE GAIN / PROT. INTAKE
		Food	Prot.					
		gm	*gm*	*gm*	*gm/gm*	*gm/gm*	*gm/gm*	*cal/gm*
W	High-protein, 85.5%	285.5	49.7	91.1	0.32	1.83	0.26	4.87
Y	High-protein, 40.4%	242.0	25.1	56.4	0.23	2.23	0.34	6.22
Z	Low-protein, 85.5%	292.9	30.4	79.9	0.27	2.63	0.37	7.62

Critical nutrients[1] per 100 gm of diet

	LYSINE	TRYPTOPHAN	NICOTINIC ACID	ZEIN	OTHER PROTEINS
	mg	*mg*	*mg*	*gm*	*gm*
85% high-protein-corn diet	522	140	7.1	5.3	12.1
40.4% high-protein-corn diet	392	96	6.1	2.5	7.9
85.5% low-protein-corn diet	463	106	7.2	1.5	8.9

[1] Assay methods are described by Flynn et al. ('54).

and Z contain the same amount of corn protein, and the same amount of total protein, therefore the protein efficiency of the high-protein corn was lower than that of low-protein corn. One would conclude that a unit of high-protein corn is superior to a unit of low-protein corn, although a unit of protein from low-protein corn is superior to a unit of protein from high-protein corn. The explanation of these observations is shown in the assays for critical nutrients. Ration W contains the largest quantities of lysine and tryptophan, and ration Y contains the smallest. The largest gain in weight per unit of protein consumed was obtained with Ration Z.

SUMMARY

1. Rats grew slowly on both a high-protein and a low-protein corn diet and an attempt was made to identify the limiting factors in each. The high-protein corn diet contained 15% of protein and the animals which consumed it gave a slight response to tryptophan. They gave responses that were larger, and approximately equal, when the supplement was either nicotinic acid or lysine. In each of these trials the response fell short of statistical significance.

2. When nicotinic acid and lysine were added simultaneously to the high-protein corn diet the response was increased again, but the combination of tryptophan and lysine gave a still larger increase. The response to each of these combinations was statistically significant. The rats did not grow more rapidly on a combination of nicotinic acid and tryptophan than on nicotinic acid alone.

3. The low-protein corn diet contained 7% of protein, and the rats which consumed it grew more slowly than did those on the high-protein corn diet. They gave no response to single additions of either tryptophan or nicotinic acid, or to the combination of tryptophan and nicotinic acid. The response to lysine alone did not reach statistical significance. The response to lysine and nicotinic acid was not more marked than to lysine alone.

4. The response when both lysine and tryptophan were added to the low-protein corn diet was highly significant. In the samples of corn studied, lysine was the first limiting factor, and tryptophan was the second.

5. Under our experimental conditions threonine was not a limiting factor in the high-protein corn. This point was not investigated in low-protein corn.

6. The biological value of the protein in low-protein corn was superior to that of the protein in high-protein corn. However, per unit of weight, the high-protein corn was superior to low-protein corn.

7. As a source of protein for the rat, casein is superior to the protein mixture in corn, even though it is supplemented with lysine and tryptophan.

LITERATURE CITED

DOBBINS, F. A., J. L. KRIDER, T. S. HAMILTON, E. B. EARLEY AND S. W. TERRILL 1950 Comparison of high- and low-protein corn for growing-fattening pigs in dry lot. J. Animal Sci., *9:* 625–633.

EGGERT, R. G., M. J. BRINEGAR AND C. R. ANDERSON 1953 The quality of protein of normal and high protein corn for growing swine. Ibid., *12:* 282–290.

FLYNN, L. M., M. S. ZUBER, D. H. LEWEKE, R. B. GRAINGER AND A. G. HOGAN 1954 Relation between protein content of corn and concentration of amino acids and nicotinic acid. Cereal Chem., *31:* 217–228.

HOGAN, A. G. 1917 Corn as a source of protein and ash for growing animals. J. Biol. Chem., *29:* 485–493.

MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1922 Studies on experimental rickets. XXII. Conditions which must be fulfilled in preparing animals for testing the anti-rachitic effect of individual foodstuffs. Johns Hopkins Hosp. Bull., *33:* 296–302.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1952 The relationship between the protein content of corn and the nutritional value of the protein. J. Nutrition, *48:* 461–476.

MITCHELL, H. H., AND D. B. SMUTS 1932 The amino acid deficiencies of beef, wheat, corn, oats, and soybeans for growth in the white rat. J. Biol. Chem., *95:* 263–281.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32:* 459–465.

ROSE, W. C., L. C. SMITH, M. WOMACK AND M. SHANE 1949 The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids in vivo. J. Biol. Chem., *181:* 307–316.

ROSS, C. V., U. S. CARRIGUS, T. S. HAMILTON AND E. B. EARLEY 1954 Comparing high-, medium-high-, and low-protein corn for fattening lambs. J. Animal Sci., *13:* 433–437.

SAUBERLICH, H. E., WAN-YUIN CHANG AND W. D. SALMON 1953 The amino acid and protein content of corn as related to variety and nitrogen fertilization. J. Nutrition, *51:* 241–250.

———— 1953 The comparative nutritive value of corn of high and low protein content for growth in the rat and chick. Ibid., *51:* 623–635.

THE FATE OF UREA IN GROWING PIGS [1,2,3]

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The value of urea as a partial substitute for protein in the ration of swine remains controversial. Forty years ago, it was reported that pigs could utilize urea (Grafe, '13; Piepenbrock, '27); however, this has not been confirmed by others (Abderhalden and Lampe, '13; Braude and Foot, '42; Hanson and Ferrin, '55).

Similarly, in rats, discrepant results of the feeding value of urea appeared in the literature (Kriss and Marcy, '40; Lardy and Feldott, '49; Rose et al., '49). Recently, Bloch ('46) reported that small concentrations of tagged isotopes were found in the body proteins and urinary ammonia of rats after the feeding of N^{15}-labeled urea. Also the studies on metabolism of C^{14}-labeled urea in mice (Leifer et al., '48) and in cats (Kornberg et al., '51) have shown that urea is not a stable end product since it breaks down in the body. At least 2.5% of the urea N^{15} was metabolized in cats when administered parentally (Davies and Kornberg, '50).

The presence of urease in the gastric mucosa of both ruminants and non-ruminants, including humans, rats, cats

[1] Journal paper No. J – 2763 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 930.

[2] After this manuscript was prepared, the following report appeared in abstract: Dekker, E. E., 1955, Urea as a source of nitrogen for biosynthesis of amino acids. Fed. Proc., *14:* 201.

[3] This investigation was supported in part by a research grant through Dr. James Waddell of the E. I. du Pont de Nemours and Company, Wilmington, Delaware.

and pigs, supports the view that urea after administration enters into the intermediary metabolism (Luck and Seth, '25; FitzGerald, '50). Further related observations were reported by Kornberg and Davies ('52, '55). One of their conclusions was that gastric urease is of bacterial origin since the enzymic hydrolysis of C^{14}-labeled urea in cats was abolished after treating the animals with sufficient amounts of antibiotics and sulfaguanidine.

The present investigation was undertaken in order to study the metabolism of urea in the growing pig, using N^{15}-labeled urea by oral administration in conjunction with a low protein ration.

EXPERIMENTAL

Three pairs of littermate crossbred pigs weighing about 35 lb. each were used. They were pair-fed a corn-soybean oil meal ration which contained 13.3% of protein and was fortified with vitamins and minerals. A 7-day adjustment period was followed by a three-day experimental period in individual metabolism cages. During each of the three days on experiment, two pigs received 300 mg of regular urea orally while their littermates received the same amounts of N^{15}-labeled urea in capsules twice daily at intervals of 12 hours. The 5th pig was fed only one dose of labeled urea; its littermate, 5 doses (table 1). Feces and urine were collected during the experimental period. All 6 animals were sacrificed in the morning of the 4th day. Live weights, weights of liver and kidneys were recorded. Samples of blood, liver, kidneys and ham muscle were collected for analysis.

Methods of separation of protein and non-protein nitrogen in the blood and other tissues were similar to those used by Watson et al. ('49). The nitrogen was determined by the method of Rittenberg et al. ('39). The N^{15} concentrations were measured with the mass spectrometer.

RESULTS

The nitrogen balance data for the 6 pigs during the three-day experimental period are shown in table 1. The average

nitrogen retention was about 48%. The results of the determination of N^{15} in both the protein and non-protein nitrogen fractions of liver, kidneys, and blood plasma; muscle protein nitrogen; urine and fecal nitrogen are shown in table 2.

The concentration of N^{15} found in the fractions of the two control animals on the basal ration, namely, pigs 691 and 700, were about 0.004 atom% in excess. This value was close to the 0.0035% found in the sample of feed with regular urea (table 2) and this indicates the normal variation of the N^{15}/N^{14} ratio in the animals compared with that of atmos-

TABLE 1

Summary of nitrogen balances for different treatments

PIG NO.	TREATMENT		FINAL BODY WT.	INTAKE N	URINE N	FECAL N	N RETENTION
	Regular urea	N^{15} urea					
	mg	*mg*	*lb.*	*gm*	*gm*	*gm*	%
691	6 × 300 [1]	0	35.5	67.04	20.79	15.89	45.29
690	0	6 × 300 [1]	37.0	67.23	27.49	11.34	42.25
700	6 × 300	0	30.5	71.51	19.42	15.06	51.77
701	0	6 × 300	33.5	72.67	22.88	16.45	45.88
720	5 × 300	1 × 300 [2]	34.5	81.13	25.82	15.82	48.67
721	1 × 300 [2]	5 × 300	40.0	81.13	22.40	17.00	51.44

[1] Six doses of 300 mg each.

[2] The single dose of N^{15} urea for pig 720 and the single dose of normal urea for pig 721 were given in the evening of the second day.

pheric nitrogen. For pigs 690 and 701 which received N^{15}-labeled urea the enrichment of liver and blood protein with N^{15} was about 0.02 atom% in excess which agrees with the values reported in other species (Bloch, '46; Kornberg and Davies, '52). The incorporation of N^{15} with the tissue proteins of the kidneys was about 0.013 atom% in excess. The isotope concentrations in the non-protein nitrogen portions of livers and kidneys were higher than those in the protein portions; whereas the largest difference between the two fractions was found in the blood. Muscle protein showed the smallest N^{15} concentration.

TABLE 2

Concentration of N^{15} in the protein- and non-protein-nitrogen fractions of liver, kidneys, blood plasma, muscle, urine and feces

(Results expressed as atom % excess)[1]

SOURCE	FRACTION	CONTROL PIGS		TEST PIGS			
		No. 691	No. 700	No. 690	No. 701	No. 721	No. 720
Liver	Protein N	0.0035	0.0055	0.0180	0.0240	0.0150	0.0075
	NPN	0.0040	0.0050	0.0285	0.0405	0.0120	0.0095
Kidneys	Protein N	0.0050	0.0050	0.0120	0.0165	0.0085	0.0070
	NPN	0.0050	0.0035	0.0245	0.0345	0.0115	0.0050
Blood plasma	Protein N	0.0040	0.0035	0.0185	0.0215	0.0160	0.0080
	NPN	0.0045	0.0045	0.0690	0.0950	0.0175	0.0085
Muscle (ham)	Protein N	0.0040	0.0025	0.0075	0.0085	0.0050	0.0040
Urine	Total N	0.0030	0.0030	0.3790	0.3700	0.3735	0.0555
Feces	Total N	0.0045	0.0060	0.0175	0.0260	0.0310	0.0110
Feed with regular urea		0.0035					
N^{15} labeled urea		15.0600					

[1] Normal abundance of Iowa air 0.365 atom %.

Sensitivity of mass spectrometer was 0.2%.

The concentrations of N^{15} of the various fractions in tissue constituents and excreta of pig 721 were all smaller than those obtained from pigs 690 and 701. This is to be expected since this pig received 300 mg less labeled urea than the other two. The effect of a single dose of 300 mg of tagged urea was shown by the results of pig 720. While the values were quite low, almost all of them were slightly higher than those of the control animals.

TABLE 3

Distribution of N^{15} in pig 690

FRACTION	NITROGEN	N^{15} CONCENTRATION	N^{15} RECOVERED
	gm	*% excess*	*% of intake*
Urine total N	27.49	0.3790	82.36
Urine urea N	21.79	0.4401	75.81
Urine ammonia N	1.65	0.1290	1.68
Urine non-urea non-ammonia N	4.05	0.0100	0.32
Fecal N	11.34	0.0175	1.57
Liver, kidneys and blood nitrogen proteins	.....	.....	3.58

The recovery of N^{15} in the urine and feces of two test pigs, and their distributions are shown in table 3. These values agree well with the results of Kornberg and Davies ('52). The small yet significant amounts of N^{15} found in the non-urea and non-ammonia nitrogen of urine indicated the fraction of administrated urea which was metabolized in the body.

SUMMARY

This investigation was conducted to study the metabolism of urea in growing pigs using N^{15}-labeled urea by oral administration in conjunction with a 13.3% protein ration. Six pigs were used. During the three days on experiment, 4 pigs received different amounts of N^{15}-labeled urea while the

two control pigs received regular urea. All 6 animals were sacrificed on the 4th day. The results of the determination of N^{15} in both the protein and non-protein nitrogen fractions of liver, kidneys and blood plasma; muscle protein nitrogen, urine and fecal nitrogen are presented. There is a small but definite amount of the administered urea incorporated into the body protein.

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LITERATURE CITED

ABDERHALDEN, E., AND A. E. LAMPE 1913 Über den Einfluss von per os verabreichtem Harnstoff auf den Stickstoffwechsel beim Schweine. Ztschr. Physiol. Chem., *84:* 218.

BLOCH, K. 1946 The metabolism of l (+) arginine in the rat. J. Biol. Chem., *165:* 469.

BRAUDE, R., AND A. S. FOOT 1942 Wartime rations for pigs. J. Agr. Sci., *32:* 70.

DAVIES, R. E., AND H. L. KORNBERG 1950 The metabolism of N^{15}-labeled urea in cat. Biochem. J., *47:* viii.

FITZGERALD, O. 1950 The metabolism of urea in animal tissues. Ibid., *47:* ix.

GRAFE, E. 1913 Über Stickstoffansatz bei Fütterung kleiner Eiweissgaben und grösserer Mengen von Ammoniaksalzen und Harnstoff. Ztschr. Physiol. Chem., *84:* 69.

HANSON, L. E., AND E. F. FERRIN 1955 The value of urea in a low protein ration for weanling pigs. J. Animal Sci., *14:* 43.

KORNBERG, H. L., AND R. E. DAVIES 1952 The metabolism of subcutaneously injected N^{15} urea in the cat. Biochem. J., *52:* 345.

——— 1955 Gastric urease. Physiol. Rev., *35:* 169.

KORNBERG, H. L., R. E. DAVIES AND D. R. WOOD 1951 Proc. 1st Isotopes Techniques Conf. Oxford.

KRISS, M., AND L. F. MARCY 1940 The influence of urea ingestion on the nitrogen balance and energy metabolism of rats. J. Nutrition, *19:* 151.

LARDY, H. A., AND G. FELDOTT 1949 The net utilization of ammonium nitrogen by the growing rat. J. Biol. Chem., *179:* 509.

LEIPER, E., L. J. ROTH AND L. H. HEMPELMANN 1948 Science, *108:* 748.

LUCK, J. M., AND T. N. SETH 1925 The physiology of gastric urease. Biochem. J., *19:* 357.

PIEPENBROCK, L. 1927 Das Problem des Eiweissersatzes durch Harnstoff bei der Tierernährung. Fortschr. Landen., *2:* 650.

RITTENBERG, D., A. S. KESTON, F. ROSEBURY AND R. SCHOENHEIMER 1939 Studies in protein metabolism. II. The estimation of nitrogen isotopes in organic compounds. J. Biol. Chem., *127:* 291.

ROSE, W. C., L. C. SMITH, M. MOMACK AND M. SHANE 1949 The utilization of the nitrogen and ammonium salts, urea and certain other compounds in synthesis of non-essential amino acids in vivo. J. Biol. Chem., *181:* 307.

WATSON, C. J., W. M. DAVIDSON AND J. W. KENNEDY 1949 The nutritive value of nitrogenous compounds for ruminants. II. The formation of body protein from urea labeled with the isotope N^{15}. Sci. Agri., *29:* 185.

EFFECT OF RIBOFLAVIN AND CHOLINE DEFICIENCIES ON WATER METABOLISM IN RATS

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In previous publications (Guggenheim and Hegsted, '53; Guggenheim, '54a, b) it was reported that rats maintained on a diet deficient in protein or in thiamine exhibit a delayed diuretic response to a water load. Pitressin was found to exert a more powerful and longer lasting effect on deficient animals than on controls receiving a normal diet either ad libitum or in restricted amounts. Thiamine-deficient rats injected with pitressin excreted larger amounts of the hormone than did normal or pair-fed controls. Experiments carried out *in vivo* and *in vitro* showed that the ability of the liver to inactivate pitressin was impaired in thiamine deficiency. In rats maintained on a diet deficient in pyridoxine and pantothenic acid, an equally delayed diuretic response to a water load could be observed. But in contrast to protein- and thiamine-deficient animals, pitressin evokes the same response, both in rats suffering from lack of either pyridoxine or pantothenic acid, and in well-nourished controls. As could be demonstrated, the ability of the liver to inactivate pitressin is not impaired in these nutritional disorders, and pitressin was thus found not to be involved in this derangement of water metabolism. Cortisone and adrenocorticotropic hormone (ACTH), however, were shown to increase the amount of urine excreted by pyridoxine- or pantothenic acid-deficient rats. It was, therefore, concluded that the water retention observed in these latter two vitamin deficiencies is due to an

inadequate stimulation of the adrenals by the adrenocorticotropic hormone of the anterior pituitary.

There exist two physiological mechanisms regulating water metabolism, which may be impaired in nutritional deficiencies: the pitressin inactivating capacity of the liver and the anterior pituitary-adrenal system. This paper presents the results obtained with two other vitamin deficiencies both of which cause a delayed diuretic response to a water load, namely riboflavin and choline. However, whereas the water retention in riboflavin deficiency appears to be due to an

TABLE 1

Composition of diets[1]

INGREDIENT	CONTROL	HIGH FAT	LOW FAT
	%	%	%
Casein, vitamin-free	18	8	8
Cornstarch	36.5	36.5	41.5
Glucose	36.5	31.5	41.5
Coconut oil	5	5	5
Lard	..	15	..
Salt mixture[2] (No. 2 U.S.P. XIII)	4	4	4

[1] The diets were supplemented with the following vitamins in milligrams per 100 gm of ration: Thiamine 0.2, riboflavin 0.3, pyridoxine 0.1, calcium pantothenate 1.6, niacin 5.0, and choline chloride, 100. Each rat received 100 I.U. vitamin A and 4 I.U. vitamin D twice weekly.

[2] No. 2, U.S.P. XIII.

insufficient production or secretion of ACTH or both, in the choline-deficient organism, delayed diuresis seems to be the result of an inadequate destruction of the antidiuretic hormone of the posterior pituitary.

METHODS

Young male rats, weighing 40 to 50 gm, were fed one of the diets indicated in table 1. For development of riboflavin deficiency, this vitamin was omitted from the control diet. In the study of choline deficiency, two different choline-free low-protein diets (8% casein) were employed, one having a low (5%) and the other a high (20%) fat content. The diet

of the respective control rats was supplemented with 0.1% of choline-chloride as indicated in tables 1 and 3.

Crude liver fat was determined according to Official Methods of A.O.A.C. ('50).

The hormone preparations used were: pitressin [1], cortisone acetate [2] and ACTH [3]. They were injected subcutaneously after dilution with saline.

RESULTS

Riboflavin deficiency

The growth of rats on the riboflavin-deficient diet was greatly stunted. This was observed as early as the second week on the diet. The appearance of porphyrin whiskers and alopecia could be seen at the end of the second month. After 8 to 12 weeks on the diet a water load amounting to 8% of the body weight was administered to the animals by stomach tube. Thirty minutes after the administration of the water load, urine collection was begun and continued for three hours.

Table 2 shows that rats maintained on the riboflavin-deficient diet excreted significantly less urine during the first three hours after administration of a water load than did the corresponding control animals This decreased urine excretion is not due to the diminished food intake resulting from the deprivation of riboflavin, since it has been shown previously that even severe starvation has no retarding influence on the excretion of a water load (Guggenheim, '54a).

In order to elucidate the mechanism of the suppressed diuresis in riboflavin deficiency the following experiments were carried out. Aqueous liver extracts, prepared from riboflavin-deficient rats as previously described (Guggenheim, '54a), were incubated with pitressin. The liver-pitressin

[1] Parke, Davis and Company.
[2] Cortogen, Schering Corporation.
[3] ACTHAR, Armour Laboratories.

preparations were injected into normal hydrated rats and the subsequent urinary excretion measured during three hours. Tables 3 (experiment 12) and 4 show that rats, injected with pitressin which had been incubated with liver extract from deficient animals, did not exhibit a stronger anti-diuresis than rats which were treated with pitressin after incubation with liver extracts prepared from the control group. These experiments indicate that the delayed diuresis in riboflavin-deficient rats is thus not due, as in the case of

TABLE 2

Per cent excretion of a water load by choline and riboflavin deficient rats

DIET	NO. OF RATS	TREATMENT	MEANS AND STANDARD ERRORS		
			1 hr.	2 hr.	3 hr.
Control	50	....	45 ± 1.8	68 ± 1.9	76 ± 2.2
Choline deficient (high fat)	10	....	25 ± 5.9	56 ± 5.5	78 ± 3.2
Riboflavin deficient	15	....	37 ± 2.7	48 ± 2.9	54 ± 3.3
Riboflavin deficient	14	Cortisone	52 ± 2.2	65 ± 2.8	75 ± 3.2
Riboflavin deficient	14	ACTH	50 ± 5.3	69 ± 7.3	77 ± 2.2

P [1]			
Control vs. choline deficient	0.001	0.05	0.6
Control vs. riboflavin deficient	0.02	0.001	0.001
Riboflavin deficient untreated vs. cortisone treated	0.001	0.001	0.001
Riboflavin deficient untreated vs. ACTH treated	0.05	0.01	0.001

[1] Probability that the observed difference is due to chance.

thiamine deficiency, to a diminished ability of the liver to inactivate the antidiuretic hormone.

In a second series of experiments avitaminotic rats were hydrated as described above and injected subcutaneously with either cortisone acetate (3 mg per rat, 90 minutes before water load) or ACTH (4 mg per rat, 90 minutes before water load). Urine was again collected as described, and the results obtained are summarized in table 2.

It can be seen that both cortisone and ACTH restore the diminished diuresis in the avitaminotic rats to normal. The

difference in urine excretion between treated and untreated rats proved to be statistically highly significant. In this connection it should be mentioned that these hormones have no diuretic effect in normal animals (Guggenheim, '54b).

It would appear, therefore, that the diminished water ex-

TABLE 8

In vitro inactivation of pitressin by liver of rats on deficient diets

Percent excretion of a water load by normal rats following injection of pitressin previously incubated with liver extract. Means and standard error.

EXP.	DIET	SUPPLEMENT	NO. OF RATS	LIVER FAT	NO. OF TESTS	2 hr.	3 hr.
				%		%	%
1	Control	....	16		70	37 ± 1.5	65 ± 2.6
2	Low-fat, choline deficient	....	15	8.8 ± 1.0	79	23 ± 2.2	44 ± 2.9
3	Low-fat, choline deficient	Choline	11	5.3 ± 0.6	51	36 ± 1.4	58 ± 2.5
4	Low-fat, choline deficient	Vitamin B_{12} (60 μg/kg)	6	8.5 ± 1.1	34	36 ± 2.4	58 ± 1.9
5	Low-fat, choline deficient	Vitamin B_{12} (60 μg/kg) + choline	6	5.2 ± 1.2	32	37 ± 1.9	54 ± 2.4
6	High-fat, choline deficient	....	17	14.8 ± 1.7	59	23 ± 1.8	52 ± 2.9
7	High-fat, choline and caloric deficient	....	6	7.6 ± 0.5	28	36 ± 2.9	65 ± 3.2
8	High-fat, choline deficient	Choline	12	5.7 ± 0.2	45	41 ± 1.9	71 ± 2.2
9	High-fat, choline deficient	Aureomycin (50 mg/kg)	8	13.0 ± 2.4	36	31 ± 2.7	66 ± 2.3
10	High-fat, choline deficient	Vitamin B_{12} (60 μg/kg)	8	12.8 ± 2.4	35	28 ± 2.1	57 ± 2.9
11	High-fat, choline deficient	Vitamin B_{12} (1000 μg/kg)	12	12.5 ± 1.1	37	38 ± 1.9	64 ± 1.8
12	Riboflavin deficient	....	7		32	40 ± 2.1	58 ± 4.0

cretion in riboflavin-deficient rats stems from a lack of cortisone-like substances. This lack does not result from an inability of the avitaminotic adrenals to produce or to secrete the hormone nor to an impaired response of the organism to the hormone. The underlying cause of the derangement seems to be rather a diminished production or secretion of the adrenocorticotropic hormone of the anterior pituitary.

TABLE 4

Statistical analysis of table 3

EXPERIMENTS COMPARED	LIVER FAT		URINE EXCRETION			
			2 hr.		3 hr.	
	t	P	t	P	t	P
1 vs. 2			5.2	0.001	5.4	0.001
1 vs. 6			6.0	0.001	3.3	0.001
1 vs. 12			1.2	0.2	1.5	0.1
1 vs. 3			0.5	0.6	1.9	0.1
1 vs. 8			1.6	0.1	1.8	0.1
2 vs. 3	3.0	0.01	5.0	0.001	3.6	0.001
2 vs. 4	0.2	0.8	3.9	0.001	4.0	0.001
2 vs. 5	2.1	0.05	4.8	0.001	2.6	0.01
6 vs. 7	4.5	0.001	3.8	0.001	3.0	0.01
6 vs. 8	5.4	0.001	6.9	0.001	5.2	0.001
6 vs. 9	0.6	0.6	2.5	0.01	3.8	0.001
6 vs. 10	0.8	0.4	1.8	0.1	1.7	0.1
6 vs. 11	1.2	0.3	5.8	0.001	3.5	0.001

Choline deficiency

The effect of choline deficiency on water metabolism was tested with two diets, one containing 20% fat and the other only 5%. Both diets were low in protein (table 1). Young rats were kept on the respective diets for 8 weeks. During this time their weight increased by 30 to 50 gm.

A water load imposed on rats kept on a high-fat choline-deficient diet was excreted with some delay (table 2). Experiments with livers from choline-deficient rats showed an impaired inactivation of pitressin (table 3, experiments 2 and 3). Supplementation of both high- or low-fat, choline-deficient diets with choline restored the ability of the liver

to destroy pitressin (experiments 3 and 8). Thus, livers of rats on a choline-supplemented low-protein diet show approximately the same degree of pitressin inactivation as livers of rats kept on a full protein (18%) diet. The water retention and impaired ability of the liver in our choline-deficient rats is, therefore, not the result of protein deficiency.

This impaired function of the liver may be due to the accumulation of fat in the liver in choline deficiency or to some other biochemical lesion accompanying lack of choline. We studied, therefore, the effect of other nutritional factors on fat content and pitressin inactivation by the liver of choline-deficient rats. Six rats were fed the high-fat, choline-deficient diet but in restricted quantities. The weight increase of these rats during 8 weeks amounted to 5 to 15 gm only; the crude fat content of their livers was significantly reduced, and extracts prepared from these livers inactivated pitressin to a significantly higher degree than those of control rats fed the same diet ad libitum (experiment 7). In further experiments these choline-deficient diets were supplemented with either vitamin B_{12} (60 and 1000 μg per kg of ration) or aureomycin [4] (50 mg per kg of ration). None of these supplements, however, diminished the fat contents of the livers. On the other hand, the pitressin-inactivating capacity of the liver was significantly increased by the addition of vitamin B_{12} to the low-fat diet and, when added at a level of 1000 μg per kg of ration, was effective with the high-fat diet as well. A similar effect was observed with aureomycin in a high-fat choline-deficient diet.

It appears, therefore, that the antilopotropic effect of choline deficiency is not necessarily accompanied by an impairment of the pitressin-inactivating capacity of the liver.

DISCUSSION

Our experiments demonstrate that riboflavin-deficient rats show a delayed diuretic response to a water load. As in the

[4] Aureomycin was kindly supplied by Lederle Laboratories Division.

case of pyridoxine and pantothenic acid deficiencies (Guggenheim, '54b), the water retention does not appear to be due to an impairment of the ability of the liver to destroy the antidiuretic hormone of the posterior pituitary, since livers of deficient rats inactivate pitressin as fast as those of well-nourished controls. Furthermore, normal diuresis could be restored by cortisone or ACTH. It may thus be concluded that the cause of the deranged water metabolism in riboflavin deficiency is due to a lack of production or secretion of cortisone-like substances due to an insufficient stimulation by ACTH.

A similar inability of riboflavin-deficient rats to excrete a water load and the restoration of impaired diuresis to the normal by adrenal cortical hormones has been described by Gaunt, Liling and Mushett ('46). Wickson and Morgan, and more recently Forker and Morgan ('54), exposed young normal, pair-fed and riboflavin-deficient rats to a simulated altitude of 20,000 feet for 24 hours and examined their blood sugar levels, liver and muscle glycogen. Under these conditions normal and pair-fed animals, but not the deficient rats, exhibited the usual degree of gluconeogenesis. The administration of adrenal cortical extract or of cortisone before the anoxia test restored the glucogenetic function completely. The authors conclude that the rôle of cortisone in permitting or initiating the gluconeogenesis resulting from the stress of anoxia in riboflavin deficiency appears to establish adrenocortical failure as part of the deficiency syndrome.

The mechanism of water retention in choline deficiency seems to be different. Livers of rats on choline-deficient diets are less able to destroy the antidiuretic hormone than are livers of control rats. As in protein (Guggenheim and Hegsted, '53) or thiamine (Guggenheim, '54a) deficiency, diminished inactivation of the antidiuretic hormone appears to be the cause of the deranged water metabolism in rats suffering from lack of choline. Lloyd and Loewy, as reported by Lloyd ('52), were also able to demonstrate that the liver of choline-deficient rats has a markedly decreased ability to

inactivate pitressin. Water retention resulting in severe edema has been observed by Engel ('48) and Alexander and Engel ('52) in rats after prolonged feeding of diets low in protein and choline. Control animals receiving the same diets but supplemented with choline had no visible edema. In our experiments, too, the diminished pitressin-inactivating capacity of the liver of rats kept on low-protein and choline-free diets was due to the lack of choline and not to the low-protein content of the diet. It is, however, interesting to note that caloric restriction imposed on rats fed a low-protein, choline-deficient diet diminished the fat content of their livers and restored their ability to inactivate pitressin.

Vitamin B_{12} was found to improve the capacity of the liver of choline-deficient rats to inactivate pitressin without, however, reducing its fat content. It seems to be established that vitamin B_{12} controls the synthesis of labile methyl groups and exerts, therefore, a sparing action on dietary choline. (For recent reviews see Smith, '54, and Jukes and Williams, '54). Some authors (György and Rose, '50; Best, Lucas and Ridout, '54; Drill, '54), however, were unsuccessful in their attempts to demonstrate a lipotropic action of the vitamin. This discrepancy may be explained by the relatively high doses of vitamin B_{12} which are necessary for this effect to be obtained. György ('51) reported a lipotropic effect in rats on a low-protein, high-fat diet with 20 μg of vitamin B_{12} per day, whereas smaller doses (0.5 μg) were without any influence.

Vitamin B_{12} has been found to alleviate hepatic injuries due to carbon tetrachloride (Popper, Koch-Weser and Szanto, '49; Hove and Hardin, '51). The studies of Stern, Taylor and Russell ('49) may provide an explanation for the liver-protecting effect of vitamin B_{12}. These workers, noting the relationship of vitamin B_{12} to the formation of desoxyribosides, investigated the effect of this vitamin upon nucleic acids in the rat as measured by the concentration of basophilia in the liver tissue. It was found that rats which were deficient in vitamin B_{12} show few or no liver basophilia, whereas those

receiving vitamin B_{12} had a considerable number of cytoplasmatic basophilia in their liver cells. A similar mechanism may be the cause underlying the restoration of the ability of the liver to inactivate pitressin.

It has been observed that aureomycin exerts a lipotropic effect (György, Stokes, Goldblatt and Popper, '51; Baxter and Campbell, '52). This finding could not be confirmed by us. The antibiotic restored, however, the pitressin-inactivating function of the liver of rats on a choline-deficient diet. A similar beneficial effect on liver function of aureomycin and other antibiotics, incorporated into a necrogenic yeast diet, has been reported by György, Stokes, Smith and Goldblatt ('50). These authors postulated that the cause of this effect lies in the growth inhibition of toxin-producing bacteria in the lower intestine.

Leslie and Ralli ('47) using a diet containing 8% of casein and 38% fat showed a reduced diuretic response to water administration. With our high-fat diet, however, containing only 20% of fat, no impairment of pitressin inactivation by the liver was found, provided choline was included in the diet. The correlation between fatty changes in the liver and the response to water administration has been studied by Heller and Blackmore ('53). No such correlation was found. Similarly, in our experiments the supplementation of our high-fat, low-choline diet with aureomycin and vitamin B_{12} had, whilst improving the pitressin inactivating function of the liver, no effect in reducing its fat content.

SUMMARY

1. Rats maintained on a diet deficient in riboflavin or in choline exhibit a delayed diuretic response to a water load.

2. *In vitro* experiments showed that the ability of the liver to inactivate pitressin is not impaired in riboflavin deficiency. Injection of cortisone or ACTH, which are without any effect on the diuresis of normal hydrated rats, increased urine excretion in deficient animals.

3. Livers of choline-deficient rats maintained on either a low- or high-fat diet, possess a diminished pitressin-inactivating ability. Caloric deficiency imposed on rats kept on a choline-deficient, high-fat diet reduced the fat content and simultaneously improved the pitressin-inactivating capacity of the liver. Vitamin B_{12} and aureomycin likewise restored the impaired ability of the liver to destroy pitressin; they had, however, no lipotropic effect.

LITERATURE CITED

ALEXANDER, H. D., AND R. W. ENGEL 1952 The importance of choline in the prevention of nutritional edema in rats fed low protein diets. J. Nutrition, *47:* 361.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official Methods of Analysis, 7th ed.

BAXTER, J. H., AND H. CAMPBELL 1952 Effects of aureomycin on renal lesions, liver lipid and tissue choline in choline deficiency. Proc. Soc. Exp. Biol. Med., *80:* 415.

BEST, C. H., C. C. LUCAS AND J. H. RIDOUT 1954 The lipotropic factors. Ann. New York Acad. Sci., *57:* 646.

DRILL, V. A. 1954 Lipotropic effects of vitamin B_{12} and other factors. Ann. New York Acad. Sci., *57:* 654.

ENGEL, R. W. 1948 Anemia and edema of chronic choline deficiency in the rat. J. Nutrition, *36:* 739.

FORKER, B. R., AND A. F. MORGAN 1954 Effect of adrenocortical hormone on the riboflavin deficient rat. J. Biol. Chem., *209:* 303.

GAUNT, R., M. LILING AND C. W. MUSHETT 1946 Disturbance of water metabolism in vitamin deficiencies and effects of adrenal cortical hormones. Endocrinology, *38:* 127.

GUGGENHEIM, K. 1954a Effect of posterior pituitary hormone on water metabolism in thiamine deficiency. Metabolism, *3:* 44.

———— 1954b Studies on water metabolism of pyridoxine and pantothenic acid deficient rats. Endocrinology, *55:* 156.

GUGGENHEIM, K., AND D. M. HEGSTED 1953 The effect of desoxycorticosterone and posterior pituitary hormone on water and electrolyte metabolism in protein deficiency. Am. J. Physiol., *172:* 23.

GYÖRGY, P. 1951 Effects of antibiotics and vitamin B_{12} in cirrhosis and necrosis of the liver. Liver Disease, A Ciba Foundation Symposium, London, p. 81.

GYÖRGY, P., AND C. S. ROSE 1950 Effect of vitamin B_{12} on experimental hepatic injury. Proc. Soc. Exp. Biol. Med., *73:* 372.

GYÖRGY, P., J. STOKES, H. GOLDBLATT AND H. POPPER 1951 Antimicrobial agents in the prevention of experimental dietary hepatic injury (necrosis, cirrhosis) in rats. J. Exp. Med., *93:* 513.

GYÖRGY, P., J. STOKES, W. N. SMITH AND H. GOLDBLATT 1950 Studies on the use of aureomycin in hepatic disease. II. The effect of aureomycin on experimental dietary hepatic necrosis. Amer. J. Med. Sci., *200:* 6.

HELLER, H., AND K. E. BLACKMORE 1953 Absence of correlation between fatty changes in the liver and impairment of water diuresis in protein-deficient mice. Brit. J. Nutrition, *7:* 349.

HOVE, E. L., AND J. O. HARDIN 1951 Effect of vitamins E, B_{12} and folacin on CCl_4 toxicity and protein utilization in rats. Proc. Soc. Exp. Biol. Med., *77:* 502.

JUKES, T. H., AND W. L. WILLIAMS 1954 Relation of vitamin B_{12} to the metabolism of choline and methionine, in Sebrell, W. H., and R. S. Harris, The Vitamins, *1:* 432. Academic Press Inc., New York.

LESLIE, S. H., AND E. P. RALLI 1947 The effect, in rats, of high fat diet on the renal excretion of water and antidiuretic substances. Endocrinology, *41:* 1.

LLOYD, C. W. 1952 Some clinical aspects of adrenal cortical and fluid metabolism. Recent Progress in Hormone Res., *7:* 469.

POPPER, H., D. KOCH-WESER AND P. B. SZANTO 1949 Protective effect of vitamin B_{12} upon hepatic injury produced by carbon tetrachloride. Proc. Soc. Exp. Biol. Med., *17:* 688.

SMITH, L. 1954 Water-soluble vitamins, Part I. Ann Rev. Biochem., *23:* 245.

STERN, J. R., M. W. TAYLOR AND W. C. RUSSELL 1949 Relation of vitamin B_{12} to liver basophilia. Proc. Soc. Exp. Biol. Med., *70:* 551.

WICKSON, M. E., AND A. F. MORGAN 1946 The effect of riboflavin deficiency upon carbohydrate metabolism in anoxia. J. Biol. Chem., *162:* 209.

SURGICAL REMOVAL OF THE CECUM AND ITS EFFECT ON DIGESTION AND GROWTH IN RABBITS[1]

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ONE FIGURE

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According to Huang, Ulrich and McCay ('54) and Thacker and Brandt ('55), the rabbits' practice of reingesting that portion of its excreta, referred to as "soft" feces, leads to a greater efficiency of food utilization. Employing chromic oxide technique in digestion studies with collared and non-collared animals, these workers reported an improvement in utilization of protein and dry matter in rabbits allowed to practice coprophagy. Eden ('40), Harder ('49), Huang et al. ('54) found cecal contents to be very similar to the soft feces. Huang et al. ('54) suggested that the soft feces were residues of cecal contents that passed through the large intestine too rapidly to lose much water.

In this laboratory the successful removal of the cecum from rabbits has been accomplished. A study of the effect of cecectomy on production of soft feces, on digestion coefficients, and on growth and ultimate pathology has been made and is reported in this paper. These findings go a step further in establishing the function of this organ.

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EXPERIMENTAL

Animal and diets. California-white rabbits, 6 weeks or older, were used. They were maintained on diet E-9, the composition of which is given in table 1. Extra potassium was included, in view of the high potassium requirements of rabbits (Hove and Herndon, '55a).

TABLE 1

Composition of diet E-9

Casein (methanol extracted)	25.0%
Cornstarch	33.8%
Cellulose [1]	10.0%
Vitamin Pre-mix in sucrose [2]	5.0%
Salts [3]	5.0%
Cod liver oil	1.0%
Lard	19.0%
$KHCO_3$	1.2%
α-Tocopherol acetate	10 mg/week

[1] "Non-Nutritive Fibre" of General Biochemicals, Inc.

[2] Vitamins added to give the following levels in micrograms per gram of diet: thiamine, riboflavin, pyridoxine, 5 each; Ca pantothenate, 30; i-inositol, 200; niacin, 40; 2-methyl-1,4-naphthoquinone, 2; vitamin B_{12}, 0.05; folacin, 2; biotin, 0.1; choline chloride, 2000.

[3] Salt mixture no. 5 (Salmon, W. D., J. Nutrition, *33:* 155, 1947).

Cecectomy. The cecum was removed, as a rule, when the animal was at least 8 weeks old. The operational procedure was as follows:

The rabbits selected for surgery were starved for a period of 24 hours prior to the operation. Usually two operations were performed in one day. Saline-dextrose solution was administered subcutaneously (about 50 ml per animal) once at three hours and again at one hour prior to time of operation. This administration seemed to be a necessary and critical step in order to prevent the onset of shock that otherwise resulted in the death of the animal during or closely following the operation. The use of sulfa drugs in the diet should be avoided. In our experience dietary sulfaquanidine (0.1% level for 5 days) caused anemia, prolonged clotting time,

brittle capillaries and fatal hemorrhage during operational procedures. Aseptic techniques were adhered to as closely as possible. The general anesthesia was sodium nembutal, administered intravenously, and procaine, which was applied to the area of incision as a local anesthetic.

An incision two inches in length was made up the midline of the shaven abdomen of the rabbit, beginning at a point approximately one and three-fourths inches anterior to the

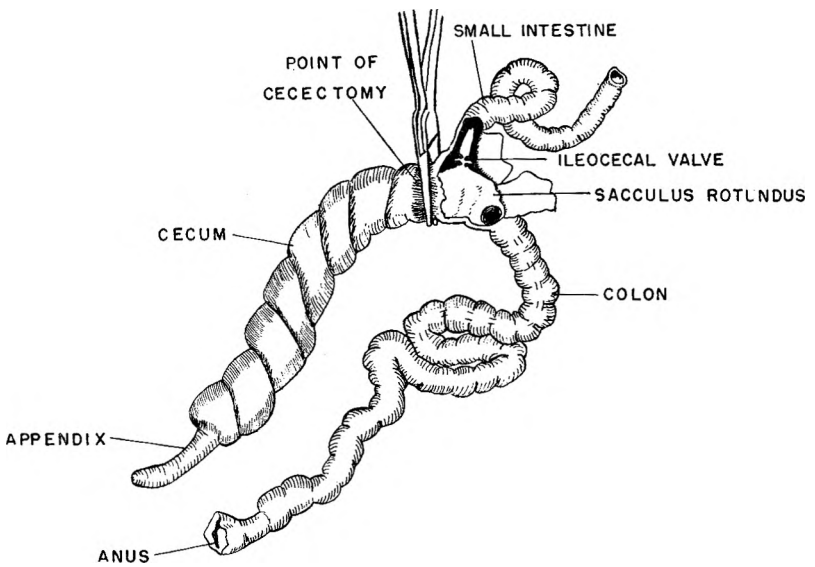

Fig. 1 Sketch showing the anatomical features of the junction of the small intestines with the cecum and colon of rabbits. Cecectomy made at clamp.

line of the hind legs. The fingers of a gloved hand were used to lift the cecum through the incision and onto a saline moistened towel that covered one side of the abdomen. The exposed intestines were kept moist and warm by means of saline-soaked gauze sponges.

The blood vessels supplying the cecum and appendix were tied off, and the cecum and its appendix were freed to the point where the small intestine joins the cecum. At this point the cecum was clamped off by means of a pair of mos-

quito forceps (fig. 1) and the body of the cecum severed free adjacent to the nose of the forceps on the side toward the appendix. A Parker-Kerr stitch was used to seal the stump. The protecting sponges were removed, loose blood clots rinsed free with warm saline and the sealed stump returned to the abdominal cavity.

The incision was closed by means of a continuous end-over-end type stitch, closing the peritoneum and muscle layer with one set of stitches and the skin layer with another set. The closed incision was painted with tincture of merthiolate and bandaged with heavy flannel.

Another 50 ml of saline-dextrose solution were administered subcutaneously, and 50,000 units of penicillin injected intramuscularly, and this antibiotic was administered periodically for the first two days after operation. The animal was then placed in a tilted cage, head downward. This position has proved to be helpful in preventing shock. Diet E-9 was offered to the animals.

Successful operations have been performed on 5 rabbits to date, and these have lived for extended periods, up to 9 months after cecectomy, with no external evidence to indicate that growth and development had not been entirely normal. Approximately 15 other rabbits have been operated on but died of shock during or shortly after the operation. Most of these deaths occurred before sufficient surgical skill was developed. One animal died 6 weeks after the operation probably because of adhesions, but in no other cases were adhesions a problem.

In experiments designed to determine the effect of cecectomy on the digestibility of feed, fecal and feed samples were analyzed for nitrogen, crude fat, fiber, and riboflavin as described in "Official Methods of Analyses of the A. O. A. C. — 1950." Sodium, potassium, and calcium were measured by emission spectrophotometric procedures, using the Beckman DU Flame photometer.

RESULTS AND DISCUSSION

Effect of cecectomy on growth and pathology

After the operation the rabbits lost weight for about two days after which they began to eat and grow. The growth rate was as high as 30 gm per day for short periods on diet E-9, although the average rate of gain was about 22 gm daily over 50-day periods. Normal rabbits fed the diet gained approximately 27 gm per day. Cecectomized animals always remained outwardly normal in appearance. After extended periods following cecectomy, however, enlargement of the adrenal and colon were observed. The kidneys were enlarged, pale, and slightly scarred. All other organs appeared normal. The enlargement of the colon may have been in compensation for the absence of the cecum.

Effect of cecectomy on soft feces production

The data apparently have indicated that rabbits with their cecum removed did not excrete typical soft feces, nor did they practice coprophagy. There was no change in the type or amount of excreta when such animals were stanchioned to prevent coprophagy. Although two types of pellets were excreted by cecectomized animals, the difference was principally one of color. One of the types of feces was the hard, dry, white pellets, uniform in size, customarily excreted by all rabbits. On diet E-9, normal rabbits generally excreted a white pellet. In addition to these white pellets, the cecectomized rabbits voided at periods throughout the day a dark, irregular-shaped, moist pellet that was identical in color to the true soft feces voided by normal rabbits. The usual attributes of soft feces, such as high protein, and the glossy, tough, mucous covering characteristic of the true soft feces were absent; furthermore, the irregular-shaped, brown feces of the cecectomized rabbit were excreted singly and not in the grape-like clusters of the true soft feces.

Chemical analyses on the various types of fecal excretion of rabbits are given in table 2. The two types of feces ex-

creted by cecectomized rabbits were nearly identical in composition. The color of the brown feces was due to bile pigments. The hard, white fecal pellets had no bile pigments. The absence of bile from part of the feces may indicate that bile was secreted periodically into the intestinal contents, or that bile was reabsorbed from that portion of the feces that remained long in the colon and became the hard-type pellet. The latter hypothesis would imply that the ileocecal valve governed the flow of nutrients into the colon. The regulation of flow would serve to retain food above the ileocecal valve,

TABLE 2

Chemical composition of feces of normal and cecectomized rabbits[1]

	NORMAL RABBITS			CECECTOMIZED RABBITS		
	Hard	Soft	Calcul. total	Hard	Soft	Calcul. total
Dry fecal wt. daily, gm	9.4	3.2	(12.6)	7.5	4.5	(12.0)
Fecal protein, % dry	14.80	41.86	(21.5)	18.15	22.34	(20.0)
Fecal fiber, % dry	36.9	25.7	(34.0)	35.9	30.3	(33.6)
Fecal fat, % dry	2.69	3.36	(2.9)	4.18	6.88	(5.2)
Fecal ash, % dry	14.8	14.3	(14.6)	13.5	13.2	(13.4)
Bile pigments (Erlich's test)	0	+		0	+	
Color	white	brown		white	brown	

[1] Stanchioned animals, 24-hr. collection periods, on diet E-9 modified to contain 10% fat (instead of 20%).

while food below this point was being subjected to thorough action by the sacculated large intestine, which absorbs out nutrients, bile, water, and which allowed a certain degree of fiber digestion to proceed.

The small amount of food substances accumulated in the lower part of the small intestine above the ileocecal valve would remain there until the time of emptying of the colon by defecation. Upon defecation, the reduced pressure could cause the ileocecal valve to open and the accumulated food substance to flow rapidly through the colon without absorption of bile or water and to be voided as soft feces. That soft feces are excreted immediately after the major voiding of

hard feces was shown by Thacker and Brandt ('55). The absence of any tendency for the cecectomized rabbits to reingest their excreta may have been due to the fact that the peculiarly high nutritive value of soft feces was missing in such animals, and it would probably have been of little profit to the animal if it had consumed that portion of excreta, which corresponded to the soft feces. Possibly some chemical, physical, or neural motivator peculiar to the cecum induces the rabbit to consume excreta as it is voided. Cecectomy apparently disrupted this mechanism. On the basis of these findings, it is evident that the cecum is the origin of the major portion of the soft type feces common to normal rabbits, while some small portion is contributed, possibly in the manner suggested above, by the lower portion of the small intestine immediately above the ileocecal valve.

Effect of cecectomy on digestibility of feed

Values are given in table 3 for feed intake and fecal excretion of two cecectomized and two normal rabbits. These animals were maintained in metabolism cages, and were not stanchioned or collared. Thus the normal rabbits consumed their soft feces completely, while the cecectomized rabbits did not. Data were collected during two periods of three days each.

The data on fecal composition in table 3 indicate that the rabbits without a cecum excreted more than 5 times the normal amount of sodium and potassium, and also slightly more protein and fat. The crude fiber content of the feces showed no significant change from the normal value. The fecal compositions in this series differed somewhat from those reported in table 2, due perhaps to the different fat levels of the diet.

The data on apparent digestibility (table 3) indicate a slightly lowered efficiency in digestion of total nutrients, protein and fat in cecectomized rabbits. It is apparent that sodium and potassium are poorly utilized by animals with no cecum. This may indicate disorder in water metabolism

in such animals. However, reingestion of soft feces and not cecal absorption seems to account for most of the increased food utilization in normal rabbits permitted to practice coprophagy.

It is evident from apparent digestibility coefficients (table 3) that the digestion of crude fiber was not influenced by the absence of the cecum. These data do not support the usual

TABLE 3

Effects of cecectomy on growth, fecal composition, and digestibility coefficients in rabbits

(Averages of two animals per group, each run for two periods of 3-day collection. Diet E-9 fed to animals for 30 days prior to test and 40 days after operation.)

CATEGORY OF INTEREST	FEED ANALYSIS	FECES ANALYSIS		APPARENT DIGESTIBILITY COEFFICIENTS	
		Cecectomized [1]	Normal [1]	Cecectomized	Normal
Body weight (gm)		1360	1165		
Gain (gm/day)		22.9	35.7	81.2	84.9
Feed/day (gm)		58.4	61.0		
Dry feces/day (gm)		11.37	9.17		
Protein, %	25.1	13.28	7.84	89.8	94.0
Fat, %	20.6	11.60	6.90	87.7	94.8
Ash, %	5.5	11.49	13.45	60.3	62.0
Fiber, %	10.3	33.77	40.72	37.8	39.1
Calcium, %	0.68	0.84	1.11	77.7	75.6
Potassium, %	1.12	0.746	0.123	87.1	98.5
Sodium, %	0.358	0.333	0.059	82.3	97.7
Riboflavin (μg/gm)	3.00	6.35	4.25		

[1] Non-stanchioned animals.

concept that the cecum is the major site of fiber digestion. Several workers have shown that the crude fiber of cecal contents is lower than that of colon contents (Huang et al., '54). This has been assumed to indicate digestion of fiber in the cecum. However, it would be equally valid to assume that crude fiber was preferentially excluded from entrance into the cecum in the first case. Thacker and Brandt ('55) were unable to show increased cellulose digestion in rabbits fed roughage diets and allowed to consume their soft feces, as

compared with similar rabbits stanchioned to prevent coprophagy. It is evident that a certain amount of fiber digestion will occur in rabbits independent of any cecal function.

A function often attributed to the cecum assumes it to serve as a type of rumen in which various B vitamins are synthesized. High levels of B vitamins were demonstrated in the soft feces of rabbits by Kulwich et al. ('53) and Olcese et al. ('48), and assumed to have originated in the cecum. However, the cecum apparently is unable to synthesize sufficient amounts of certain of the vitamins, since Wooley ('45) was able to produce a niacin deficiency, and Hove et al. ('54) found the rabbit to be very susceptable to a dietary deficiency of choline. Typical vitamin B_6 deficiency, with acrodynia, anemia and xanthurenic acid excretion, has been produced in rabbits on vitamin B_6-deficient diets (Hove and Herndon, '55b).

It may be concluded that the rabbit, in its digestive apparatus, more nearly resembles other monogastric animals, and may not be compared closely with the bovine and other ruminants. Supporting this view, Olcese and Pearson ('48) showed that urea can not replace dietary proteins in rabbits.

SUMMARY

1. Surgical removal of the cecum of rabbits has been successfully accomplished, and 5 animals lived for periods up to 8 months. The growth rate of cecectomized rabbits averaged 22 gm per day when fed a synthetic-type diet with 10% fiber and 20% fat. At post mortem there was enlargement of the colon and adrenal glands. Normal growth rate of rabbits was between 25 and 30 gm per day over a 50-day period on this diet.

2. Rabbits without ceca continued to excrete two types of feces, differentiated on the basis of color, bile pigments, and shape. However, the so-called "soft" feces of these animals were not in grape-like clusters; they did not have the tough gelatinous coating nor the high protein, low fiber contents typical of normal soft feces. Coprophagy was not practiced.

3. The digestibility of fiber (cellulose) was equal in both normal and in cecectomized rabbits. Sodium and potassium utilization were greatly lowered in cecectomized rabbits; protein and fat digestibility were somewhat lower, while ash and calcium were unchanged. It is concluded that, functionally, the digestive apparatus of rabbits is similar to other monogastric animals.

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LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official Methods of Analysis. 7th Edition, Washington, D. C.

EDEN, A. 1940 Coprophagy in the rabbit: Origin of night feces. Nature, *145:* 628.

HARDER, W. 1949 Zur Morphologie und Physiologie des Blinddarms der Nagetiere. Verh. Deutch. Zool., Mainz, *2:* 95.

HOVE, E. L., D. H. COPELAND AND W. D. SALMON 1954 Choline deficiency in the rabbit. J. Nutrition, *53:* 377.

HOVE, E. L., AND J. F. HERNDON 1955a Potassium deficiency as a cause of muscular dystrophy in rabbits. J. Nutrition, *55:* 363.

———— 1955b Unpublished data.

HUANG, T. C., H. E. ULRICH AND C. M. MCCAY 1954 Antibiotics, growth, food utilization and the use of chromic oxide in studies of rabbits. J. Nutrition, *54:* 621.

KULWICH, R., LEE STRUGLIA AND P. B. PEARSON 1953 The effect of coprophagy on the excretion of B-vitamins by the rabbit. J. Nutrition, *35:* 577.

OLCESE, O., AND P. B. PEARSON 1948 Value of urea in the diet of rabbits. Proc. Soc. Exp. Biol. Med., *69:* 377.

OLCESE, O., P. B. PEARSON AND B. S. SCHWEIGERT 1948 The synthesis of certain B-vitamins by the rabbit. J. Nutrition, *35:* 577.

THACKER, E. J., AND C. S. BRANDT 1955 Coprophagy in the rabbit. J. Nutrition, *55:* 375.

WOOLEY, J. G. 1945 Niacin deficiency in rabbits and response to tryptophan and niacin. Proc. Soc. Exp. Biol. Med., *65:* 315.

THE EFFECT OF A PREOPERATIVE PREFERENCE FOR SUGAR OVER SALT UPON COMPENSATORY SALT SELECTION BY ADRENALECTOMIZED RATS [1]

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ONE FIGURE

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Richter ('42) has observed prompt changes in the appetitive choices of laboratory rats following the onset of a variety of nutritional disturbances. He has noted among other observations, that parathyroidectomized rats exhibit increased calcium intakes, that thiamine depleted rats ingest greater quantities of thiamine and that bilaterally adrenalectomized rats show an accentuated preference for sodium chloride. From these studies Richter concludes ('42, '47) that whenever a need develops for one of the items of diet the laboratory rat immediately develops a specific hunger for the required substance. Thus, if the required substance is available, it is assumed that a life-long condition of least possible tissue stress resulting from the deficiency is assured.

Alternative to Richter's viewpoint, Harris et al. ('33) and Scott and Verney ('47) propose that compensatory appetitive choices do not necessarily occur immediately following the development of a nutritional need and may be prevented from occurring at all. These investigators found that a previously

[1] This study was carried out under the direction of Dr. Robert B. MacLeod, while the author was a Public Health Post-Doctoral Research Fellow at Cornell University.

beneficial preference for a foodstuff by rats depleted for a specific dietary item will continue for the foodstuff after the required item has been removed. This preference may be so strong as to prevent a shift in preference to an equally accessible food to which the required item has been added. Harris et al. ('33) and Scott and Verney ('47) have not eliminated the possibility, however, that the first compensatory food preference may develop both immediately and to an adequate degree. Perhaps it is for this reason that Richter ('47) holds that the findings of these investigators may not be taken as a refutation of his position.

In the present experiment the possibility that development of an original compensatory appetitive choice can be delayed or prevented is investigated. It is predicted that if a strong and irrelevant preference for sugar over salt is established in normal rats, the preference will continue following bilateral adrenalectomy and will serve to check the typical post-operative increase in salt intake.

EXPERIMENTAL

Ninety-six male rats, Wistar strain, aged 65 to 75 days at the beginning of the experiment, were utilized. Twenty-four rats were randomly entered into each of 4 groups. Each rat was maintained in an individual cage and was given unlimited quantities of a diet, devised by the experimenter, which, while otherwise nutritionally adequate, contained 0.007% sodium and 0.1% potassium. Two inverted water bottles were attached to each of the cages. Both bottles and attached drinking tubes were cleaned daily and were independently varied in position according to the schedules of two random order series.

Condition 1. Two groups were given continuous access to an 8% by weight sugar solution and to a 1.2% by weight salt solution for an 18 to 20-day preoperative period. The solvent in each solution was distilled water. The rats quickly demonstrated a strong and stable preference for the sugar solution. During the interval from the 18th through the 20th day, one

group was bilaterally adrenalectomized, and the other group was bilaterally sham adrenalectomized. Immediately following operation, 20 survivors from each group were again given the solutions for an additional 15-day period. Fluid intakes and body weights were recorded daily during the postoperative period.

Condition 2. The other two groups had access to distilled water which was available in the inverted bottles during the 18 to 20-day preoperative period. Then, one group was bilaterally adrenalectomized, and the other was bilaterally sham adrenalectomized. Immediately after operation, an 8% sugar and a 1.2% salt solution were initially presented to 20 survivors in each group. As with the other two groups, fluid intakes and body weights were recorded daily for a 15-day postoperative period.

RESULTS AND DISCUSSION

Tests for the significance of differences between groups were made through application of Festinger's non-parametric technique ('46) to the average daily intakes of salt solution.

Figure 1 indicates, in accordance with Richter's observations, that the animals of the adrenalectomized group, lacking preoperative experience with either salt or sugar, rapidly developed and maintained a marked preference for the salt solution. Table 1 shows that the quantity of salt solution ingested by this group during the 15-day postoperative period was significantly greater at the 0.01 level of confidence than that ingested by any of the other three groups. That this preference served to reduce tissue need is seen in the fact that, like the members of the sham adrenalectomized groups, all the rats in this group survived during the postoperative period.

The results obtained from the group given preoperative salt and sugar experience contrast markedly with those obtained from the group lacking such experience. As shown in table 1, not only was the intake of salt solution by the animals of this group significantly lower than it was for those without

access to sugar or salt solution before adrenalectomy, but it was insignificantly different from that of either of the two sham adrenalectomized groups.

Like the animals in the sham adrenalectomized groups, those in the adrenalectomized group, given opportunity to develop

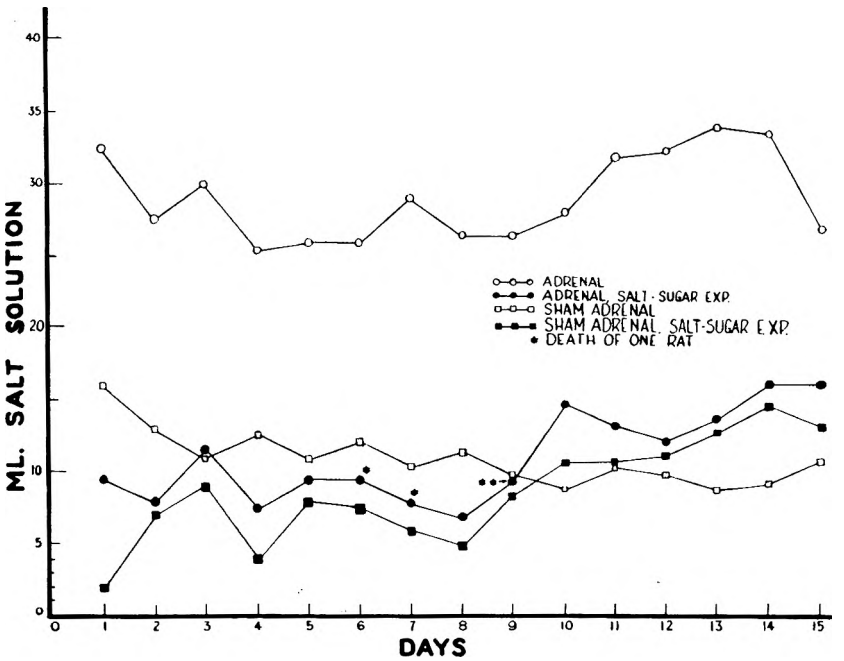

Fig. 1 Mean daily intakes for the 15-day postoperative period of a 1.2% salt solution by each of 4 groups of laboratory rats.

TABLE 1

Festinger's non-parametric test of the significance of differences between mean salt intakes for two groups of adrenalectomized and two groups of sham adrenalectomized rats during the 15-day postoperative period [1]

GROUPS	ADRENALECTOMIZED (Salt-sugar exp.)	SHAM ADRENALECTOMIZED	SHAM ADRENALECTOMIZED (Salt-sugar exp.)
Adrenalectomized	7.5	7.5	7.5
Adrenalectomized (Salt-sugar exp.)	...	0.2	2.9
Sham adrenalectomized	...	...	3.1

[1] Values equal to or greater than 4.1 are significant at the 0.01 level of confidence.

a strong preference for sugar over salt prior to operation, continued to select the sugar solution and to neglect the salt solution during the postoperative period. This failure to increase salt intake following operation had serious consequences. Four of the rats in this adrenalectomized group died during the postoperative period, and the survivors exhibited marked weight losses. While the mean weights of the rats in the two adrenalectomized groups were comparable for the first three postoperative days ($t = 1.92$, 38 df), the mean weight of the 16 surviving rats which had developed a preference for sugar over salt before adrenalectomy, was significantly lower on the final three postoperative days ($t = 5.84$, 34 df) than that of the group without the preoperative salt and sugar treatments.

The failure of the group with a preoperatively established sugar preference to increase its salt intake following bilateral adrenalectomy would seem to confirm the experimental prediction. Apparently, the development of the typical postoperative increase in salt selection by adrenalectomized rats can be checked if the rats have developed a preference for sugar over salt prior to adrenalectomy. This failure can have consequences so severe as to result in the deaths of some such rats and in marked weight losses in the survivors. From this experiment, it appears, therefore, in contrast to Richter's view, that postoperative salt selection by bilaterally adrenalectomized laboratory rats may not be necessarily either immediately increased or sufficient to preserve a condition of minimal tissue stress resulting from sodium deficit.

SUMMARY

This experiment was undertaken to test the prediction that the typical postoperative increase in salt selection by adrenalectomized rats may be delayed or prevented by a preoperatively established irrelevant sugar preference.

Under the first experimental condition, two groups of laboratory rats were given continuous access to a salt and to a sugar solution for an 18 to 20-day period. Under the sec-

ond condition, two other groups were given distilled water during this period. At the end of the period, one group under the first condition and one group under the second condition were bilaterally adrenalectomized; the two remaining groups were sham adrenalectomized. All 4 groups were then given access to a salt and to a sugar solution for a 15-day period. Weights and fluid intakes were recorded daily during this interval for the members of each group.

The results appear to confirm the prediction. Laboratory rats given opportunity to establish a strong preoperative preference for sugar over salt maintain the preference subsequent to adrenalectomy and fail to manifest an increase in salt intakes. During the 15-day postoperative period, these rats (1) ingested significantly less of the salt solution than did adrenalectomized rats without the preoperatively established sugar preference and (2) ingested no more salt than did either of the sham adrenalectomized groups. As a consequence of this failure to increase salt intake, several of the rats died and marked weight losses occurred in the survivors.

LITERATURE CITED

FESTINGER, L. 1946 The significance of differences between means without reference to the frequency distribution function. Psychometrika, *2:* 102.

HARRIS, L. J., J. CLAY, F. J. HARGREAVES AND A. WARD 1933 Appetite and choice of diet. The ability of the vitamin B deficient rat to discriminate between diets containing and lacking the vitamin. Proc. Royal Soc. London, *113:* 161.

RICHTER, C. P. 1942 Total self-regulatory functions in animals and human beings. The Harvey Lecture Series, *38:* 63.

———— 1947 Biology of drives. J. Comp. Physiol. Psych., *40:* 129.

SCOTT, E. M., AND E. L. VERNEY 1947 The nature of appetites for B vitamins. J. Nutrition, *34:* 471.

VITAMIN A METABOLISM IN INFECTION

EFFECT OF STERILE ABSCESSES IN THE RAT ON SERUM AND TISSUE VITAMIN A [1,2]

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Children with rheumatic fever have low plasma vitamin A levels. With the onset of increased rheumatic activity the levels drop even further (Shank et al., '44; Leitner, '51; Wang et al., '54). Josephs ('43) and Lawrie et al. ('41) showed that blood vitamin A levels are reduced in pneumonia and that with convalescence the vitamin A levels rise well above normal. Aron et al. ('46) and Aron ('49) showed that fever, induced by physical means, causes the plasma vitamin A and carotene levels to be lowered and that the degree of lowering is directly related to the duration of fever.

Moore and Sharman ('51) found that liver vitamin A concentrations were low in patients who had abscesses or pneumonia when they died.

The purpose of this study was to determine if tissue demand or increased utilization of vitamin A during the course of infection is the reason for the lowering and what the source of vitamin A is during the rapid rise after the infection subsides.

[1] This study was supported in part by a grant from the National Vitamin Foundation.

[2] Presented before the 122nd Annual Meeting of the American Chemical Society, Atlantic City, New Jersey.

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Wendt and Weyrauch ('27), Emery and Matthews ('43), and Majumber and Wintrobe ('48) showed that injections of turpentine or of sweet almond oil into rats produced abscesses which resembled bacterial abscesses in their histologic appearance. These substances were, therefore, used to produce such abscesses in rats, and the vitamin A changes in the blood and tissues of these animals were studied.

MATERIALS AND METHODS

Mature albino male rats of the Sprague-Dawley strain weighing between 250 and 350 gm were used. All the animals were fed Purina Dog Checkers containing 8 U. S. P. units of vitamin A per gram. Control animals were given the same standard feeding as the experimental animals.

One cubic milliliter of rectified turpentine oil N. F. or sweet almond oil U. S. P. was injected subcutaneously or intramuscularly as described below.

Serum vitamin A concentrations were determined by the micro-method of Bessey et al. ('46). Liver vitamin A was determined as previously described by Kagan and Kaiser ('52).

Temperatures were taken at regular intervals on both experimental and control animals.

EXPERIMENTAL

One group of rats was injected subcutaneously with turpentine, and this was repeated one week later. The serum levels of vitamin A determined in these and in control rats at intervals thereafter are summarized in table 1.

One week after the initial injection, all the experimental animals showed a marked drop in serum vitamin A, and two days after the second injection, this was even more striking. The control animals which received no injections showed no significant change in serum vitamin A during this time.

A second group of rats (table 1) was injected three times with 1 ml of turpentine subcutaneously at weekly intervals. Serum vitamin A levels were determined before injection and one week after first injection, two and 7 days after second injection, and two days after third injection. There was a

TABLE 1

Effect of subcutaneous injection of 1 ml of turpentine in rats

Serum vitamin A — μg per 100 ml
Mean ($\pm$ standard deviation)

	NO. RATS	BEFORE INJECTION	2 DAYS AFTER 1ST INJECTION	7 DAYS AFTER 1ST INJECTION	2 DAYS AFTER 2ND INJECTION	
Exp. group 1 Two injections at weekly intervals	5	28 ($\pm$2.7)	23 ($\pm$10.5)	16 ($\pm$2.3)	10 ($\pm$5.4)	
Control	5	25 ($\pm$5.8)	26 ($\pm$1.2)	33 ($\pm$4.2)	26 ($\pm$1)	
			1 WEEK AFTER 1ST INJECTION	2 DAYS AFTER 2ND INJECTION	7 DAYS AFTER 2ND INJECTION	2 DAYS AFTER 3RD INJECTION
Exp. group 2 Three injections at weekly intervals	6	27 ($\pm$1.4)	18 ($\pm$2.7)	13 ($\pm$2.3)	19 ($\pm$3.2)	10 ($\pm$8.2)
			2 DAYS AFTER 4TH INJECTION			
Exp. group 3 Four injections at weekly intervals	6	24 ($\pm$3.3)	9 ($\pm$2.3)			
Control	6	23 ($\pm$1.5)	19 ($\pm$1.2)			

progressive decrease in vitamin A levels except when the results 7 days after the second injection are compared to those two days after the second injection. A 7 days, there thus appears to be some recovery. Two days after the third injection, however, very low levels were again observed.

Table 1 also shows the results when rats were injected subcutaneously 4 times at weekly intervals with 1 ml of turpentine. The mean serum vitamin A in these animals two days after the 9 injections was 9 μg per 100 ml (standard error, ± 3) as compared to 19 μg per 100 ml (standard error, ± 3) in the control group.

Table 2 shows the results when 4 similar injections were given intramuscularly to rats. The results are like those obtained with subcutaneous injections.

These results, therefore, show serum vitamin A changes which are similar in direction to those observed in bacterial

TABLE 2

Effect of 4 intramuscular injections of 1 ml of turpentine in rats at weekly intervals

Serum vitamin A — μg per 100 ml
Mean (± standard deviation)

NO. RATS	BEFORE INJECTION	2 DAYS AFTER 3RD INJECTION	2 DAYS AFTER 4TH INJECTION
Experimental			
5	25 (± 4)	11 (± 2.6)	9 (± 2.5)
Control			
5	24 (± 2.6)	20 (± 3.6)	24 (± 2.4)

infections in humans. Since these results might have been due to turpentine, per se, rather than to the abscesses, sweet almond oil was used in one experiment (table 3). The results obtained with this oil are similar to those with turpentine.

Table 4 shows the vitamin A concentrations in the livers of injected and control animals. The animals were sacrificed three days after the 4th injection. The mean weight of the livers of the experimental animals was not significantly different from that of the controls. However, the liver vitamin A concentration and the total vitamin A content of the livers of the experimental animals were significantly lower than those of the control animals.

Table 5 shows the vitamin A concentration in the livers of animals similarly treated except that they were given one large oral dose of vitamin A in order to determine their ability to absorb and deposit it in their livers. In these animals, there is no difference from the controls in liver vitamin A content or concentration.

Test doses of vitamin A were given to animals with abscesses and to control animals, and the effect on subsequent blood levels was studied. The test dose was 12,000 units of

TABLE 3

Effect of subcutaneous injection of 1 ml of sweet almond oil in rats two injections given one week apart

Serum vitamin A — μg per 100 ml
Mean (± standard deviation)

NO. RATS	BEFORE INJECTION	2 DAYS AFTER 1ST INJECTION	7 DAYS AFTER 1ST INJECTION	2 DAYS AFTER 2ND INJECTION
Experimental				
5	27 (± 10)	23 (± 7)	21 (± 4)	13 (± 7)
Control				
5	30 (± 8)	24 (± 7)	26 (± 10)	29 (± 10)

TABLE 4

Liver vitamin A

Three days after 4th injection of turpentine

	NO. RATS	WT. OF LIVER	LIVER VITAMIN A	
		gm ± *S.D.*	*μg/gm* ± *S.D.*	*μg/ total liver* ± *S.D.*
Experimental	6	12.0 ± 1.2	101 ± 11	1092 ± 140
Control	6	10.9 ± 1.4	131 ± 16	1406 ± 124

TABLE 5

Liver vitamin A

Three days after 4th injection of turpentine and one day after oral dose of 12,000 I.U./kilogram body wt.

	NO. RATS	WT. OF LIVER	LIVER VITAMIN A	
		gm ± *S.D.*	*μg/gm* ± *S.D.*	*μg/total liver* ± *S.D.*
Experimental	6	9.4 ± 1	206 ± 11	1950 ± 193
Control	6	10.4 ± 2	203 ± 25	2127 ± 455

vitamin A alcohol per kilogram of body weight in aqueous dispersion [4] by gavage. The vitamin A alcohol preparation, which originally contained 25,000 units/ml, was diluted before administration for more accurate measure of dosage to contain 3,000 units/ml. Groups of 5 to 6 animals were studied. Except as indicated, 1 ml of turpentine was injected subcutaneously. Blood levels were determined before the test dose and three, 6, and 24 hours later. In one group, the test dose was given two days after a single injection; in other group it was given two days after a single injection of sweet almond oil; in a third group it was given two days after a second weekly injection; and in a 4th group, it was given two days after a third

TABLE 6

Serum vitamin A

Four injections of 1 ml turpentine in rats at weekly intervals
Effect of oral dose of vitamin A alcohol — 12,000 I.U./kilogram body wt.
Dose given two days after 4th injection

	NO. RATS	SERUM VITAMIN A — μG % MEAN ± S.D.			
		Before 1st injection	Before dose	3 Hours after dose	24 Hours after dose
Experimental	5	32 ± 4	17 ± 2	168 ± 34	14 ± 9
Control	5	37 ± 5	41 ± 6	203 ± 109	48 ± 5

injection. The results of one such experiment are shown in table 6. There were no significant differences in the post-test-dose rise in blood vitamin A at the three- and 6-hour time intervals. These results suggest again that gastrointestinal absorption and liver storage were not modified by the abscesses. The levels 24 hours after the test doses in the experimental animals returned to about the pre-test dose low levels.

Vitamin A concentrations in the central purulent portion of the abscesses were also determined and compared with adjacent tissues and tissue of normal rats. No significant differences were found. The vitamin A levels in these tissues of both the experimental and control rats were very low

[4] Kindly supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

(center of abscesses 1.6 to 2.3 μg per gram, average 2.0; periphery 2.9 to 2.9; normal subcutaneous tissue 2.0 to 2.6, average 2.2).

Studies of vitamin A concentrations in other tissues, including the kidney, spleen and intestinal wall also revealed no statistically significant differences between the mean values for the experimental and control animals. However, the mean value for vitamin A concentration in the kidneys was over twice as high in the experimental as in the control group. The difference was not statistically significant because the variation between samples tested was very large.

TABLE 7

Urine vitamin A

Four injections of 1 ml turpentine in rats at weekly intervals
Urine collected for three-day periods beginning two days after each injection

	NO. RATS	3 DAYS BEFORE 1ST INJECTION		AFTER INJECTIONS	
		Urine vol.	Urine vit. A	Urine vol.	Urine vit. A
		ml/24 hrs. ± S.D.	*μg/24 hrs. ± S.D.*	*ml/24 hrs. ± S.D.*	*μg/24 hrs. ± S.D.*
Experimental	6	45 ± 10	6.4 ± 0.7	106 ± 38	42.7 ± 36.0
Control	6	52 ± 16	4.9 ± 3.3	78 ± 19	11.2 ± 5.9

The urine (table 7) also revealed much higher levels of vitamin A in some of the experimental animals as compared to levels before injection and as compared to urine levels of control animals. However, again because of the marked variation in urinary excretion of vitamin A in the individual experimental animals there was no significant difference in the mean. In humans, there is normally no detectable vitamin A in the urine whereas in pneumonia, appreciable amounts appear in some patients. The loss of vitamin A through the urine would not appear to fully explain the decreased amounts in the blood and liver in the rats in these studies.

The rats' temperatures were taken frequently. None of the experimental animals had rectal temperatures of over 37.3 ° C. and none of the control rats had temperatures over

36.9 ° C. The mean temperature of the experimental animals was 36.8° C. and that of the controls was 36.2° C. It seems unlikely that depression of serum vitamin A in the experimental animals was due to the slight elevation of temperature, but this possibility cannot be excluded in this experiment. Wang et al. ('54) found that, in rheumatic fever, the serum vitamin A levels were lowered even in the absence of fever although not to the same extent as when fever was present.

DISCUSSION

The data are analogous to those of previous reports showing that the serum vitamin A falls in the presence of infection. The fact that the liver vitamin A concentration also becomes lower suggests that there is increased demand by other tissues for vitamin A when such infection is present. The only other tissue in which a change was found was in kidney vitamin A where it was erratically increased. There also appears to be an increase in urine vitamin A. The possibility that it is lost in the urine in a possible detoxification process is suggested.

Large doses of cortisone (Clark and Colburn, '55) given to rats has been reported to result in rapid loss of liver and also of kidney vitamin A. It may be that in rats with abscesses, increased secretion of adrenal steroids under the stress of the situation may be responsible for lowering of liver vitamin A. With these abscesses, however, the kidney vitamin A if anything is increased, not decreased. Reichstéin's Compound L Acetate has been reported (Bodansky and Markardt, '51) to lower the blood and kidney vitamin A of rats and to increase liver vitamin A. These conflicting findings will probably fit into a more understandable relationship once more information is obtained.

SUMMARY

Sterile abscesses produced by single or multiple subcutaneous or intramuscular injections of turpentine or sweet almond oil in rats resulted in marked lowering of serum vitamin A concentrations. The liver vitamin A content and

concentrations also decreased. The response to oral administration of test doses was, however, normal, and the liver concentrations of vitamin A after a large oral dose did not differ from the normal control animals given similar doses. Vitamin A concentrations in the centers and peripheries of abscesses did not differ from those of normal subcutaneous tissues. As a result of the abscesses, some loss of the vitamin A occurs through the urine. Speculations as to the possible significance of these findings are discussed.

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LITERATURE CITED

ARON, H. C. S. 1949 Plasma vitamin A and its clinical significance. A review. Am. J. Dis. Children, *77:* 763.

ARON, H. C. S., R. M. CRAIG, C. J. FARMER, H. W. KENDELL AND G. X. SCHWEMLEIN 1946 Effect of elevated body temperature on plasma vitamin A and carotene. Proc. Soc. Exp. Biol. Med., *61:* 271.

BESSEY, O. A., O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ 1946 The determination of vitamin A and carotene in small quantities of blood serum. J. Biol. Chem., *166:* 177.

BODANSKY, O., AND B. MARKARDT 1951 Effect of Reichstein's Compound L Acetate on plasma, liver and kidney vitamin A. Ibid., *190:* 83.

CLARK, I., AND R. W. COLBURN 1955 A relationship between vitamin A metabolism and cortisone. Endocrinology, *56:* 232.

EMERY, F. E., AND C. S. MATTHEWS 1943 A note on cysts and abscesses induced in the rat by the injection of oils. J. Lab. Clin. Med., *28:* 1795.

JOSEPHS, H. W. 1943 Studies on vitamin A. Vitamin A and total lipid of the serum in pneumonia. Am. J. Dis. Children, *65:* 712.

KAGAN, B. M., AND E. KAISER 1952 Vitamin A concentration in the liver in the nephrotic syndrome. J. Lab. Clin. Med., *40:* 12.

LAWRIE, N. R., T. MOORE AND K. R. RAJOGOPAL 1941 The excretion of vitamin A in urine. Biochem. J., *35:* 825.

LEITNER, Z. A. 1951 Pathology of vitamin A deficiency and its clinical symptoms. Brit. J. Nutrition, *5:* 130.

MAJUMBER, D. N., AND M. M. WINTROBE 1948 Anemia of injection. IX. Influence of adrenal cortical hormone on hypoferremia and other blood changes associated with injection of turpentine. J. Lab. Clin. Med., *33:* 532.

MOORE, T., AND I. M. SHARMAN 1951 Vitamin A levels in health and disease. Brit. J. Nutrition, *5:* 119.

SHANK, R. E., A. F. COBURN, L. V. MOORE AND C. L. HOAGLAND 1944 The level of vitamin A and carotene in the plasma of rheumatic subjects. J. Clin. Invest., *23:* 289.

WANG, P., H. L. GLASS, L. GOLDENBERG, G. STEARNS, H. G. KELLY AND R. L. JACKSON 1954 Serum vitamin A and carotene levels in children with rheumatic fever. Am. J. Dis. Children, *87:* 659.

WENDT, H., AND F. WEYRAUCH 1927 Experimentelle Untersuchungen über die Wirkungsweise der Terpentinabscesse. Klin. Wchnschr., *6:* 165.

EFFECTS OF OCTACHLORONAPHTHALENE ON VITAMIN A METABOLISM IN THE RAT [1]

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Cattle suffering from hyperkeratosis (X-disease), produced by feeding chlorinated naphthalenes, have been found to have decreased concentrations of vitamin A in their blood (Hansel et al., '51; Copenhaver and Bell, '54). The specific anti-vitamin A effect of these toxic agents has been emphasized by the report of Hoekstra et al. ('54) in which no significant alterations were found in the blood carotene, niacin, or vitamins C, B_6 and B_{12} in hyperkeratotic calves. In similar animals, however, Hove ('53) found a reduction in plasma vitamin E as well as vitamin A. Although other species have been shown to be more resistant than cattle to chlorinated naphthalenes (Hoekstra et al., '54), the anti-vitamin A action of these compounds appeared to warrant a more detailed investigation in a species in which vitamin A metabolism is more easily studied. Since the completion of this study, Schoettle et al. ('55) have reported that chlorinated naphthalenes decrease the liver vitamin A of rats and hamsters.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Texas, NR 123-118. A preliminary report of this work appeared in Fed. Proc., *14:* 428 (1955).

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EXPERIMENTAL

Weanling rats of the Holtzman strain were caged individually in an air-conditioned room at 25 ± 1° C. Two vitamin A-free diets were employed, one a purified type (SAF) and the other semipurified (USP), with compositions shown in table 1. The toxic agent [1], stated by the manufacturer to contain approximately 90% of octachloronaphthalene (OCN) and 10% of heptachloronaphthalene, was mixed in powdered form into

TABLE 1

Composition of vitamin A-deficient diets

INGREDIENT	DIET SAF	DIET USP
	%	%
Purified casein	22	18
Cottonseed oil [1]	2	5
Sucrose	70	..
Corn starch	..	65
Salts 4 [2]	4	4
Dried brewers' yeast [3]	..	8
Vitamin mix [4]	2	..

[1] Wesson oil, with *dl*-α-tocopherol and vitamin D_2 added equivalent to 2 mg and 150 U.S.P. units, respectively, per 100 gm diet.

[2] Hegsted et al. ('41).

[3] Primary dried yeast, strain G, Anheuser-Busch, Inc.

[4] Supplies per 100 gm diet, in micrograms: thiamine·HCl 1000, riboflavin 1000; pyridoxine · HCl 500; calcium pantothenate 5000; niacin 2000; folic acid 200; biotin 200; 2-methyl-1,4-naphthoquinone 500; inositol 100; p-aminobenzoic acid 1000; vitamin B_{12} 0.1; plus choline chloride 100 mg. Mixture in sucrose.

the diet. The quantity of food offered was controlled so that the groups in each experiment consumed equal amounts. Commercial crystalline carotene [5] or a vitamin A concentrate [6] was administered orally in cottonseed oil, or intravenously in aqueous dispersion containing 20% Tween 40 (Bieri, '51). Vitamin A and carotene in livers, kidneys and blood serum were determined on hexane extracts of the unsaponifiable

[4] Halowax chloronaphthalene 1051, Bakelite Company, New York.

[5] Kindly supplied by Barnett Laboratories, Long Beach, California.

[6] Drisdol, containing 50,000 U.S.P. units vitamin A and 10,000 U.S.P. units vitamin D_2 per gram.

fractions by the conventional Carr-Price method. The microspectrophotometric procedure of Bessey et al. ('46) was also used in some experiments for the analysis of sera. The method of Swick and Baumann ('52) for vitamin E in liver was modified by using pyrogallol as the anti-oxidant in the saponification. Vitamin E in serum was determined by the procedure of Quaife et al. ('49); because of the lower concentration of vitamin E in rat blood than in human blood, the size of the sample was increased to 1.0 ml. For total liver lipid, the tissue was ground with anhydrous sodium sulfate and extracted with chloroform.

TABLE 2

Vitamin A in sera and combined livers and kidneys of rats ingesting octachloronaphthalene (OCN) for 22 days[1]

DIET	AV. WT. GAIN	LIVERS + KIDNEYS	SERUM[2]
	gm	*μg/gm*	*μg/100 ml*
USP	47.0	37.6 ± 7.3[3]	59.6
USP + 0.05% OCN	48.5	13.9 ± 1.1	80.4
USP + 0.20% OCN	55.5	8.4 ± 1.5	73.0
USP + 0.50% OCN	45.7	4.6 ± 0.5	97.2

[1] Six male rats per group; initial average weight 99.4 ± 0.1 gm.
[2] Average of three samples; blood of two rats pooled for analysis.
[3] Means with standard error.

RESULTS

Effect on vitamins A and E

Preliminary studies with rats on a commercial stock diet revealed that as little as 0.002% OCN in the diet for 28 days would significantly depress the vitamin A in the liver. In the first experiment, designed to study the effect of OCN on preformed vitamin A in the body, 24 rats partially depleted of the vitamin for 5 days on the USP diet were given a daily oral supplement of 994 μg of vitamin A for 10 days, in order to build up uniform stores. The animals were then grouped by weight and OCN added to the diets. Analysis of the tissues after 22 days (table 2) revealed a reduction in stored vitamin

A by the lowest concentration of OCN (0.05%) to 37% of that of the control rats ($P = <0.001$), while the highest amount of OCN (0.50%) decreased the storage to 12% of that of the controls. The vitamin A in the serum, however, was not depressed by feeding OCN but may have been increased. There was no effect of the OCN on growth rate.

While this study was in progress, Hove ('53) reported that in cattle with hyperkeratosis plasma vitamin E, as well as vitamin A, was depressed. He suggested that chlorinated naphthalenes are pro-oxidants in vivo and may exert their anti-vitamin effect in this way. In a study planned to de-

TABLE 3

Vitamins A and E in tissues of rats ingesting octachloronaphthalene (OCN) for 7 days [1]

DIET	INITIAL AV. WT.	VITAMIN A		VITAMIN E
		Liver + kidneys	Serum	Liver
	gm	*μg/gm*	*μg/100 ml*	*μg/gm*
USP	107	5.6 ± 0.6 [2]	36.3 [3]	59.1 ± 9.5 [2]
USP + 0.05% OCN	114	3.6 ± 0.4	36.9	50.1 ± 8.7
USP + 0.30% OCN	109	2.6 ± 0.3	30.4	40.6 ± 7.4

[1] Six male rats per group; given 121.5 μg carotene orally 4 days before being killed.

[2] Means with standard error.

[3] Average of three samples; blood of two rats pooled for analysis.

termine the possible co-existence of lowered vitamins A and E as a result of feeding OCN, weanling rats depleted of their vitamin A for 14 days on the USP diet were grouped and OCN added to the diet. After 4 days, when weights were 88 to 96 gm, they received a single dose of 121.5 μg of carotene in oil orally. Four days later they were sacrificed after fasting 24 hours and the tissues and sera analyzed (table 3). Vitamin A in the combined livers and kidneys was reduced in the groups ingesting OCN ($P = <0.001$ for 0.3% OCN), but there was no effect on the vitamin in the serum. Although there was an indication of a decrease in the vitamin E content of the livers of the OCN-fed rats, the differences were

not statistically significant ($P = > 0.1$ for 0.3% OCN). In a second study, two groups of young rats (three males and three females per group) which had been fed a commercial stock ration for two weeks were given the purified diet (SAF), with or without 0.3% OCN, ad libitum. After 16 days, the animals were fasted 24 hours and sacrificed. Analyses of the sera revealed no significant changes in the concentrations of vitamin A (34.6 and 29.5 μg%, average of three rats) or of vitamin E (486 ± 16 and 503 ± 30 μg%, average and standard error of 6 rats, control and OCN rats respectively). Body weight was also unaffected by the chlorinated naphthalene.

Effect on carotene utilization

Rats made deficient in vitamin A by feeding the USP diet for 22 days were grouped and the diet supplemented with OCN. Body weights at this time were 90 to 128 gm. After three days, half of the animals in each group were given a single oral dose of 131 μg of carotene in oil, and the other half were injected intravenously into the tail vein with 131 μg of carotene dispersed in water. The vitamin A and carotene found in the combined livers and kidneys 24 hours after receiving carotene are shown in table 4. In the groups receiving carotene intravenously, there were no differences between the control and OCN-ingesting rats in the amount of vitamin A formed, nor in the carotene recovered from the tissues. In the orally-treated groups, however, the OCN-ingesting rats formed only about two-thirds as much vitamin A as the controls ($P = < 0.001$).

Attempts to estimate fecal excretion of carotene were unsuccessful because the ether extracts of feces of rats ingesting OCN contained large quantities of a yellow pigment presumably derived from the OCN [7]. Quantitative separation of this substance from carotene was not successful. The pigment also could not be separated from unchanged OCN by boiling with acid or alkali.

[7] Feeding 0.1% of a mixture of hexa- and pentachloronaphthalenes (Halowax 1014) produced a similar yellow fecal extract.

TABLE 4

Effect of octachloronaphthalene (OCN) on carotene utilization by the rat as determined by tissue storage of vitamin A

DIET	NO. RATS [1]	ROUTE OF CAROTENE ADMIN.[2]	LIVER + KIDNEYS [3]	
			vitamin A	Carotene [4]
			μg, total	*μg, total*
USP	5	Intraven.	7.3 ± 0.8	9.8 ± 0.6
USP + 0.10% OCN	7	Intraven.	7.6 ± 0.7	8.3 ± 0.7
USP + 0.30% OCN	7	Intraven.	6.2 ± 0.6	8.5 ± 0.8
USP	5	Oral	9.7 ± 0.9	Trace
USP + 0.10% OCN	6	Oral	6.4 ± 1.2	Trace
USP + 0.30% OCN	6	Oral	6.2 ± 0.7	Trace

[1] All males, vitamin A-deficient, having no residual "apparent" vitamin A in their tissues.

[2] Single carotene dose of 131 μg; animals killed after 24 hours.

[3] Means with standard error.

[4] Essentially all of the carotene was in the livers.

TABLE 5

Influence of dried brewers' yeast on liver weight and lipid content as a result of feeding octachloronaphthalene (OCN) for 14 days [1]

DIET	AV. WT. GAIN	LIVER WT.	TOTAL LIVER LIPIDS [2]
	gm	*% body wt.*	*%*
SAF	40.0	3.81 ± 0.04 [3]	5.05 ± 0.21 [3]
SAF + 0.1% OCN	37.8	6.14 ± 0.32	14.14 ± 1.77
SAF + 8% yeast + 0.1% OCN	38.0	5.16 ± 0.19	6.61 ± 0.45
USP	35.2	3.74 ± 0.11	6.09 ± 0.32
USP + 0.1% OCN	29.3	5.82 ± 0.31	15.14 ± 1.83

[1] Six male rats per group.

[2] Fresh weight basis.

[3] Means with standard error.

Effect on the liver

Upon autopsy of rats ingesting OCN, it was noted that animals on the USP diet had livers which appeared normal or only slightly yellow, whereas the livers from rats on the SAF diet were uniformly mottled and light colored. It appeared that the yeast in the USP diet may have been responsible for this difference, possibly through the action of "Factor 3" of Schwarz ('51). To investigate this possibility, weanling rats were fed the two diets with or without OCN, and one group received the SAF diet with 8% of dried brewer's yeast substituted for sucrose. After 14 days, they were sacrificed following a 24-hour fast and the livers analyzed for total lipid. Histological sections were prepared using hematoxylin and eosin stain. The results in table 5 show that OCN added to either diet doubled the liver weight and liver lipid. Addition of yeast to the SAF diet did not prevent hypertrophy of the organ, but did prevent fat accumulation. Microscopic examination revealed that although the added yeast eliminated fatty infiltration of the cells, these livers showed lesions similar to those of the two groups receiving OCN alone.

DISCUSSION

It is evident that octachloronaphthalene, or perhaps a substance derived from it, produces in the rat a specific elimination of vitamin A from the liver but does not alter the vitamin in the blood. The effect on liver vitamin A is not accompanied by changes in vitamin E in this organ or in the blood. These latter observations do not support the suggestion of Hove ('53) that chlorinated naphthalenes are pro-oxidants and may act on unsaturated fat systems generally. It is apparent that the toxicity of chlorinated naphthalenes in the rat is considerably different from that in cattle, where plasma vitamin A and vitamin E, as well as liver vitamin A, are reduced.

The finding that conversion of injected carotene was not impaired by OCN, whereas the utilization of orally adminis-

tered carotene was diminished, suggests that OCN does not inhibit the actual conversion mechanism but acts by affecting the absorption or stability of the provitamin in the intestine. It is of interest that OCN, which is highly inert chemically, may be modified in the intestine, as evidenced by the yellow material found in the feces.

The effect of yeast in preventing fatty livers of rats on the SAF diet with OCN is not readily apparent. This diet contained adequate amounts of choline and tocopherol, and 22% casein. Possibly the additional protein from the yeast was responsible, since Engel et al. ('55) found that 27% of casein protected against fat infiltration due to chlorinated naphthalenes. Since a nutritional role for Schwarz's Factor 3 in adequate diets has not been demonstrated, it cannot be said that the factor was involved here. The yeast did not prevent the liver hypertrophy and microscopic lesions resulting from the OCN. A similar hypertrophy, not prevented by protein, was reported by Engel et al. ('55).

SUMMARY

The addition of 0.05 to 0.30% of octachloronapthalene to the diet of rats greatly accelerated the loss of vitamin A from the liver. There was no effect on the vitamin A or vitamin E in the blood, nor did the vitamin E in the liver change significantly. Utilization of injected carotene was not impaired by feeding octachloronaphthalene but a marked decrease in utilization occurred with carotene given per os.

Octachloronaphthalene in the diet caused the excretion in the feces of an unidentified yellow material.

The addition of 8% of brewers' yeast to a purified diet containing octachloronaphthalene prevented fatty infiltration in the liver, but did not eliminate the accompanying hypertrophy and lesions of that tissue.

LITERATURE CITED

BESSEY, O. A., O. H. LOWRY, M. J. BROCK AND J. A. LOPEZ 1946 The determination of vitamin A and carotene in small quantities of blood. J. Biol. Chem., *166:* 177.

BIERI, J. G. 1951 Stability of aqueous and oily vitamin A and carotene solutions. J. Nutrition, *44:* 327.

COPENHAVER, J. S., AND W. B. BELL 1954 The production of bovine hyperkeratosis (X-disease) with an experimentally made pellet feed. Vet. Med., *XLIX:* 96.

ENGEL, R. W., W. N. LINKOUS AND W. B. BELL 1955 Effect of protein on the toxicity of hexachloronaphthalene. J. Nutrition, *55:* 119.

HANSEL, W., K. MCENTEE AND P. OLAFSON 1951 The effects of two causative agents of experimental hyperkeratosis on vitamin A metabolism. Cornell Vet., *41:* 367.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of the chick. J. Biol. Chem., *138:* 459.

HOEKSTRA, W. G., R. E. HALL AND P. H. PHILLIPS 1954 A study on the relationship of vitamin A to the development of hyperkeratosis (X-disease) in calves. Am. J. Vet. Res., *15:* 41.

HOVE, E. L. 1953 The toxicity of tri-o-cresyl phosphate for rats as related to dietary casein level, vitamin E and vitamin A. J. Nutrition, *51:* 609.

QUAIFE, M. L., N. S. SCRIMSHAW AND O. H. LOWRY 1949 A micromethod for assay of total tocopherols in blood serum. J. Biol. Chem., *180:* 1229.

SCHOETTLE, C. E., E. F. REBER, C. C. MORRILL AND R. P. LINK 1955 Experimental production of hyperkeratosis in rats and hamsters. Am. J. Vet. Res., *16:* 183.

SCHWARZ, K. 1951 A hitherto unrecognized factor against dietary necrotic liver degeneration in American yeast (Factor 3). Proc. Soc. Exp. Biol. Med., *78:* 852.

SWICK, R. W., AND C. A. BAUMANN 1952 Chemical assay for tocopherol in animal materials. Anal. Chem., *24:* 758.

THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

XVI. A COMPARISON OF LINOLEATE AND LINOLENATE IN SATISFYING THE ESSENTIAL FATTY ACID REQUIREMENT FOR PREGNANCY AND LACTATION [1,2]

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In a recent report from this laboratory (Deuel et al., '54), it was established that optimal results in pregnancy, as judged by survival of the pups and their 3-day body weights, were obtained on fat-free diets, when the mothers were supplemented with 200 mg daily of cottonseed oil (which contains approximately 50% of linoleate) from the onset of breeding. The same amount of cottonseed oil also proved to be optimum for lactation, as judged by the survival of the pups and their body weight at 21 days. When methyl linoleate was used as the supplement in place of cottonseed oil, results comparable to the optimum obtained with cottonseed oil were reported when the daily dosage of linoleate was 80 mg. Quackenbush and co-workers ('42) reported that the requirement of rats

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[2] Contribution No. 390 of the Department of Biochemistry and Nutrition of the University of Southern California.

for the essential fatty acids during pregnancy and lactation is approximately twice that necessary for growth and for the cure of dermal symptoms. While linoleate and arachidonate were found to supply this need successfully, Quackenbush et al. ('42) reported that linolenate was completely inactive.

The negative results of Quackenbush et al. ('42) on the role of linolenic acid in pregnancy and lactation are unexpected, in view of the earlier experiments of Burr and co-workers ('32), who assigned approximately equal biopotency to linoleic and linolenic acids in overcoming the fat-deficiency syndrome. Tange ('32) also reported that these two acids possessed equal biological activity. However, a number of others have found linolenate inferior to linoleate in supplying the need of the animals for essential fatty acids. Thus, Hume and collaborators ('38) observed that linolenate possessed only one-sixth of the effectiveness of linoleate, while Martin ('39) reported the only completely negative result on linolenate. More recently, Greenberg and co-workers ('50) reported that, although linolenic acid given alone was practically without activity in restoring growth in fat-deficient rats, its effectiveness was equal to that of linoleic acid when the two were administered together. It was suggested that the linolenate required the "sparking" effect of linoleate to be effectively metabolized. In addition, linolenate has recently been shown to be practically ineffective in protecting rats from x-irradiation injury unless it was administered simultaneously with linoleate; under these conditions, a marked synergism in the protection afforded was observed (Cheng et al., '55).

In the present studies, we have investigated the relative biopotency of linoleate and linolenate separately, and in combination, on the reproductive performance in rats. The present experiments have enabled us to confirm the optimum values of linoleate for pregnancy and lactation which were previously reported.

EXPERIMENTAL

The experimental procedures were similar to those employed in our earlier experiments (Deuel et al., '54). Weanling female rats of the U.S.C. strain were maintained on a fat-free diet for 10 weeks, at which time they were divided into the several experimental groups listed in table 1. At this time they were bred with proven normal male rats from our stock colony. The composition of the basal diet was identical to that employed by Greenberg et al., ('50) and to that used in our earlier tests (Deuel et al., '54). The samples of linoleate and linolenate were pure samples of methyl and ethyl ester,[3] respectively. The amounts of ester used were calculated on the basis of the free acid and are therefore comparable. The esters were given orally by syringe.

RESULTS AND DISCUSSION

The average results are recorded in table 1.

It is apparent from the data presented in table 1 that linolenate is not as effective in supplying the essential fatty acid requirement for reproduction and lactation as is linoleate. In all groups except group 21 (fat-free diet, unsupplemented), there was 100% fertility and practically the same number of pups were cast. However, when the reproductive performance is based upon the survival at three days, the percentage of mortality is much greater in the case of the linolenate-supplemented animals, except at the highest dosage level. Thus, the comparative mortality rates of the animals fed linoleate (groups 22, 25, 28, 30), and linolenate (groups 23, 26, 29, 31), respectively, were the following at the several daily dosage levels: 10 mg, 75%, 88%; 20 mg, 41%, 88%; 40 mg, 20%, 35%; 100 mg, 16%, 9%. However, when linoleate and linolenate were given together, the response was equal to, or greater than that of the group receiving the combined total amount as linoleate. Thus, the mortality at three days was 42% for group 24 (10 mg each of linoleate and linolenate)

[3] Purchased from the Hormel Foundation.

TABLE 1

A comparison of the effect of linoleate and linolenate, alone and in combination, on pregnancy, survival, and growth of young rats from females on a fat-free diet

CATEGORY	GROUP NO.										
	21	22	23	24	25	26	27	28	29	30	31
Linoleate fed daily, mg	0	10	..	10	20	..	20	40	..	100	..
Linolenate fed daily, mg	0	..	10	10	..	20	20	..	40	..	100
Females bred	15	14	15	14	15	15	15	15	15	15	15
Litters cast, %	93.3	100	100	100	100	100	100	100	100	100	100
Litter (at birth)											
Total number of rats	99	134	127	131	146	139	152	131	143	147	139
Average number per litter	7.1	9.6	8.5	9.4	9.7	9.3	10.1	8.7	9.5	9.8	9.3
Litter (at 3 days)											
Total number of rats	3	34	15	76	86	17	145	105	83	123	126
Total litters represented	1	6	2	11	12	3	15	15	12	15	14
Average weight per rat, gm	4	5.4	6.6	5.8	5.6	4.6	5.8	6.2	5.7	6.2	6.4
Litter (at 21 days)											
Total number of rats	..	7	5	59	57	..	91	73	5	77	66
Total litters represented	..	2	1	11	12	..	15	15	3	12	13
Average weight per rat, gm	..	29.5	30.0	26.5	26.4	..	27.2	28.9	30.2	29.6	28.4
Mortality (0–3 days)											
Total	96	100	112	55	60	122	7	26	50	24	13
%	97	75	88	42	41	88	5	20	35	16	9
Mortality (3–21 days)[1]											
Total	3	20	8	9	9	15	9	10	65	13	30
%	100	74	62	13	14	100	9	12	93	14	31

[1] Litters were reduced to 7 at three days.

as compared with 41% for group 25 (20 mg linoleate). Likewise, the figure for group 27 (20 mg each of linoleate and linolenate) was 5% as compared with 20% for group 28 (40 mg linoleate).

Insofar as lactation is concerned, the pattern is quite similar to that observed above. Thus, with the exception of the lowest dosage, at which the number of rats which survived three days was too small to allow for a valid conclusion at the end of 21 days, the 21-day mortality rate was lower when linoleate was used as a supplement than when linolenate served in this capacity. The percentage of mortality at three to 21 days, omitting groups 22 and 23 (lowest dosage), was as follows for animals supplemented with linoleate and linolenate, respectively: 20 mg, 14%, 100%; 14 mg, 12%, 93%; and 100 mg, 14%, 31%. When linoleate and linolenate were given together, the response was the same as that elicited by the same total quantity of linoleate.

In the present tests, the lactation response was as satisfactory when linoleate was given at the 40 mg dosage (group 28) as when administered at 100 mg daily (group 30). The response was also equivalent to that obtained with an 80 mg daily supplement in our earlier tests (Deuel et al., '54). However, when one takes into consideration the total number of rats born, and the average number of pups per litter, one must conclude that the overall reproductive performance is better in the group receiving the highest linoleate supplement (group 30). When no supplement of essential fatty acids was given (group 21), only three of the 99 rats cast survived for three days, and none survived for the 21-day period. This result is similar to that obtained in our earlier tests (Deuel et al., '54).

SUMMARY

The relative biopotencies of linoleate and of linolenate for supplying the essential fatty acid requirement in pregnancy and lactation have been compared in the rat. Linolenate was found to be less effective than linoleate at all levels studied.

However, when mixtures of linoleate and linolenate were given, the potency of linolenate was found to be equal if not greater than that of an equivalent total dose of linoleate. The synergism of linoleate and linolenate, previously reported by this laboratory for growth and protection against x-irradiation injury, has been shown to exist under the conditions of pregnancy and lactation as well.

LITERATURE CITED

BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acids essential in nutrition. III. J. Biol. Chem., *97:* 1.

CHENG, A. L. S., T. GRAHAM, R. B. ALFIN-SLATER AND H. J. DEUEL, JR. 1955 The effect of fat level of the diet on general nutrition. XV. Comparison of the protective effect of linoleic acid and linolenic acid against multiple sublethal doses of x-irradiation in the rat. J. Nutrition, *55:* 647.

DEUEL, H, J., JR, C. R. MARTIN AND R. B. ALFIN-SLATER 1954 The effect of fat level of the diet on general nutrition. XII. The requirement of essential fatty acids for pergnancy and lactation. Ibid., *54:* 193.

GREENBERG, S. M., C. E. CALBERT, E. E. SAVAGE AND H. J. DEUEL, JR. 1950 The effect of fat level of the diet on general nutrition. VI. The interrelation of linoleate and linolenate in supplying the essential fatty acid requirement in the rat. Ibid., *41:* 473.

HUME, E. M., L. C. A. NUNN, I. SMEDLEY-MACLEAN AND H. H. SMITH 1938 Studies of the essential unsaturated fatty acids and their relation to the fat-deficiency disease of rats. Biochem. J., *32:* 2162.

MARTIN, G. J. 1939 Studies of fat-free diets. J. Nutrition, *17:* 127.

QUACKENBUSH, F. W., F. A. KUMMEROW AND H. STEENBOCK 1942 The effectiveness of linoleic, arachidonic, and linolenic acids in reproduction and lactation. Ibid., *24:* 213.

TANGE, U. 1932 A study on the effects of fatty acid on nutrition. Sci. Pop. Inst. physic. chem. Research, *20:* 13.

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1956 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1956. For details of nomination procedure, write to Chairman of the Nominating Committee.

Chairman, Nominating Committee:
DR. OTTO A. BESSEY
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