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THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

THYROID FUNCTION IN THE YOUNG PIG AND ITS RELATIONSHIP WITH VITAMIN A[1,2]

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(Received for publication August 25, 1958)

Jungherr et al. ('50) observed hyperplasia of the thyroid glands in vitamin A-deficient bull calves. Johnson and Baumann ('47) found that hyperthyroidism in the rat increased the liver storage of vitamin A. This work indicated that the outcome was not due to changes in the basal metabolic rate (BMR) *per se,* but was brought about by some other physiological action of the thyroid gland. In spite of the fact that elevated BMR increased the rate of depletion of the liver vitamin A in the rat, thyroxine so enhanced the storage of vitamin A from the diet that liver concentration rose above that of controls (Johnson and Baumann, '48). Nevertheless, their study showed that changes in growth rate have a greater influence upon vitamin A utilization than changes in metabolic rate.

Wiese et al. ('48) suggested that, although rats rendered hypothyroid by thiouracil consumed their liver vitamin A at a lower rate, this was a consequence of their lower rate of growth. This was corroborated by work with dogs (Arnrich, '55) which further demonstrated that the hypothyroid con-

[1] Journal Paper no. J-3376 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 959.

[2] The authors wish to express gratitude to Drs. A. F. Voigt, R. M. Melampy and R. S. Allen for their helpful criticism and advice, and to acknowledge Hoffmann-La Roche, Inc., Nutley, New Jersey and Western Condensing Company, Appleton, Wisconsin for grants-in-aid and materials which partially supported this work.

dition increased the circulating vitamin A, carotene and cholesterol. Thyroxine administration to milking cows (Chanda and Owen, '51) increased the vitamin A alcohol content of the cow's milk. This suggests that thyroxine may function in the mobilization of liver reserves. In its general metabolic effect thyroxine is thought to be involved in oxidative phosphorylation (Martius, '55).

As has been shown by Missouri workers with cattle (Blincoe and Brody, '55) and many others, environmental temperature has a marked influence upon thyroid function. The Missouri group also concluded that one of the best parameters for measuring thyroid function was that of the rate at which the thyroid gland secretes radio-iodine. Other workers at Missouri (Pipes et al., '56) made use of thiouracil as a metabolic block in studying the rate of thyroid secretion by the dairy animal. They found a linear trend of log activity of the gland on time, indicating that the secretion rate was one of a constant percentage.

The work reported herein was initiated to study thyroid function in the pig and the influence of vitamin A upon this function. Recent evidence available to the authors stresses the role of the thyroid in promoting carotene absorption; yet since Jungherr et al. ('50) noticed thyroid hyperplasia in vitamin A deficiency, it was decided to investigate whether or not vitamin A influenced thyroid function. This was studied at two environmental temperatures.

EXPERIMENTAL

Experiment 779. The previous management of these animals was described by Frape et al. ('59). One pig from each of the 20 pens in the first part of this experiment was used in these tracer studies, plus an additional 4 pigs receiving the basal ration. Within each replication members of the same litter were selected.

On reaching 83 days of age, each pig received an intraperitoneal injection of a sterile saline solution of sodium

iodide^{131} at the rate of 0.4 μc per pound of body weight. Twenty hours subsequent to this, each pig received an intraperitoneaal injection of 2 gm of thiouracil [3] (Pipes, '57) and then all animals were placed on feed containing

TABLE 1

Composition of ration

Experiment 797

INGREDIENT	1 TO 4 WEEKS OF AGE	4 TO 8 WEEKS OF AGE
	lb.	*lb.*
Ground yellow corn	9.55	20.6
Sucrose	10.00	15.00
Ground oat groats	—	20.00
Dextrose	5.00	—
Dried whey (70% lactose)	2.50	10.00
Dried skim milk (low heat, spray dried)	40.00	10.00
Solvent soybean oil meal (50% protein)	17.50	15.00
Condensed fish solubles	2.50	2.5
Stabilized lard	5.00	2.0
Corn steep water	1.00	—
Dried brewers' yeast	1.00	—
Dried beet pulp	2.00	—
Calcium carbonate	0.15	0.45
Dicalcium phosphate	0.60	1.30
Iodized salt	0.50	0.50
Trace mineral mix [1]	0.20	0.15
Vitamin-antibiotic premix [2]	2.50	2.50
Totals	100.00	100.00

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

[2] Each 2.5 lb. of premix contained the following amounts of vitamins and antibiotics for pigs from 1 to 4 weeks of age: vitamin A, 250,000 I.U.; vitamin D_2, 60,000 I.U.; vitamin E, 1,000 I.U.; menadione, 0.1 gm; riboflavin, 0.032 gm; Ca pantothenate, 0.190 gm; niacin, 2.27 gm; choline chloride, 3.27 gm; vitamin B_{12}, 2 mg; folic acid, 0.06 gm; ascorbic acid, 30.0 gm; thiamine HCl, 0.2 gm; pyridoxine, 0.2 gm; *p*-aminobenzoic acid, 0.8 gm; chlortetracycline, 5.0 gm; penicillin, 2.5 gm; streptomycin, 2.5 gm.

Each 2.5 lb. of premix contained the following amounts of vitamins and antibiotics for pigs from 4 to 8 weeks of age: vitamin A, 250,000 I.U.; vitamin D_2, 60,000 I.U.; riboflavin, 0.14 gm; Ca pantothenate, 0.2 gm; niacin, 1.8 gm; vitamin B_{12}, 2 mg; folic acid, 30 mg; chlortetracycline, 5 gm.

[3] Information about dosage was received in a private communication from Dr. G. W. Pipes, University of Missouri, Columbia.

0.5 gm of thiouracil/lb. At 24-hr. intervals for the following 9 days, the activity of the glands was measured. The relative activity was estimated by a bismuth cathode Geiger tube in contact with the neck. The logarithms of the adjusted counting rates were used in the calculation of the secretion rate constant (b) for each animal. The size of the thyroid pool was assumed to be constant. Subsequent papers will take variations in this into account.

The animals were housed, two pigs per pen, under the management and environmental conditions described by Frape et al. ('59). The rations fed were also the same as those offered from three to 8 weeks of age.

Experiment 797. Six pigs from each of two litters were weaned at 7 days of age and divided by weight within litter into 4 groups of three pigs each. They were immediately injected intraperitoneally with 15 μc of sterile sodium iodide^{131} solution. Each received, 24 hours later, an intraperitoneal injection of 0.8 gm of sterile thiouracil and was immediately offered feed containing 0.5 gm of thiouracil/lb. for 9 days. At 7 weeks of age each pig was again injected with 28 μc of sterile sodium iodide^{131} solution.

Between one and 4 weeks of age the pigs received a diet that contained 40% of dried skim milk. From 4 weeks until the close of the experiment, they were offered a diet containing 10% of dried skim milk. These rations (table 1) contained 2,700 I.U. of vitamin A per pound.

RESULTS AND DISCUSSION

Experiment 779. The rate of thyroid secretion was found to be considerably greater than that reported for dairy cattle (Swanson et al., '57; Lodge et al., '57) and sheep (Henneman et al., '55; Singh et al., '56). However, the use of thiouracil as a metabolic block accounts for some of this discrepancy.

The plot of rate constants of thyroid secretion of vitamin A level at the average of the two temperatures in experiment 779 indicated a quadratic trend which proved to be significant

at $P < 0.05$ (see fig. 1). This indicates a maximum in secretion rate at 100 I.U. of vitamin A/lb. of ration. The secretion rates in pigs on both the basal and the ration containing 6400 I.U./lb. of feed were depressed. Neither the influence of temperature, nor that of the temperature x vitamin A level interaction on the rate constants proved to be statistically significant. The lack of an interaction is in line with other functions reported by Frape et al. ('59). In light of the experimental design (split plot), which purposely sacrificed accuracy on the main effect of temperature, an apparent effect of this may be part of the litter component of variability.

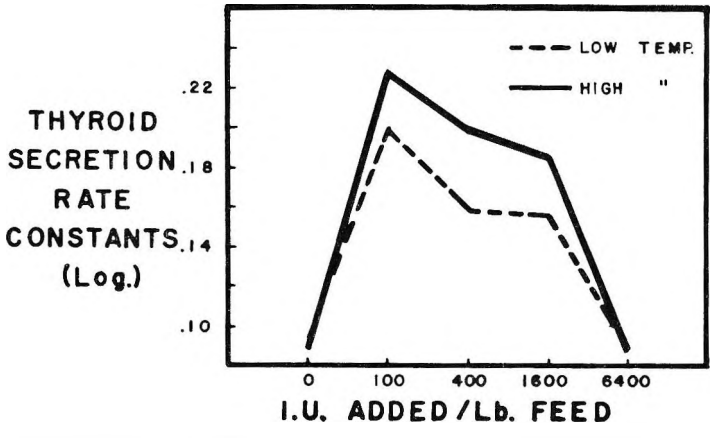

Fig. 1 Effect of vitamin A and temperature on thyroid secretion rate.

The shape of the thyroid secretion rate response curve on vitamin A level shows a similarity to that obtained for the ratio of feed to gain in the experiments reported by Frape et al. ('59). In this respect the most efficient feed conversion occurred in that region of dietary vitamin A in which the rate of thyroid secretion was greatest.

The response curve for the thyroid may be connected only indirectly with vitamin A through the effect of the latter on growth. It was hoped to clarify this point in experiment 797. The daily gains in weight are presented in table 2.

Experiment 797. The first measurement was started when the pigs were 7 days of age. These pigs were smaller than usual at this age and the initial consumption of dry feed was delayed in many of the pigs. Thyroid function was very much retarded until eating commenced. This may account to some extent for the positive correlation of 0.292 between percentage increase in body weight and rate constants between one and two weeks of age. The average rate constant of secre-

TABLE 2

Effect of dietary vitamin A and temperature on the average daily rate of gain during the 13th week of life.
Experiment 779

VIT. A ADDED PER LB. OF FEED	AVERAGE DAILY GAIN	
	High temp. 18–21°C	Low temp. 6–8°C
I.U.	*lb.*	*lb.*
0	— 0.4	— 0.3
100	1.5	1.8
400	1.9	2.0
1600	1.8	2.1
6400	1.8	2.2

tion as per cent per day was 28.1 at this time. This rose to 47.6% at 7.5 weeks of age. In experiment 779, at 13 weeks of age the average rate constants of all vitamin A levels, excluding the basals and those receiving 6400 I.U., was 32.1% per day.

The coefficients of correlation of rate constant with total gain and with percentage increase in body weight at 7.5 weeks of age were — 0.514 and — 0.411. This may be compared with a coefficient of + 0.383 for those pigs receiving from 100 through 1600 I.U. of vitamin A per pound of feed at 13 weeks in experiment 779. The relationship of thyroid secretion to growth in this experiment appears to be of a low order, suggesting that the secretion response in experiment 779 was possibly directly a consequence of the vitamin A regime.

Plotting of the response curve for the pigs at 7.5 weeks of age suggested a non-linearity of log-corrected counts on days.

This is in contrast to other reported work, where thiouracil was used. In other studies reported where this compound also played a part, the secretion rates were low and any deviation from linearity would be small and difficult to detect, considering the experimental error. Analysis of variance of our data showed that the assumption of a quadratic regression significantly reduced the variation of the observed values

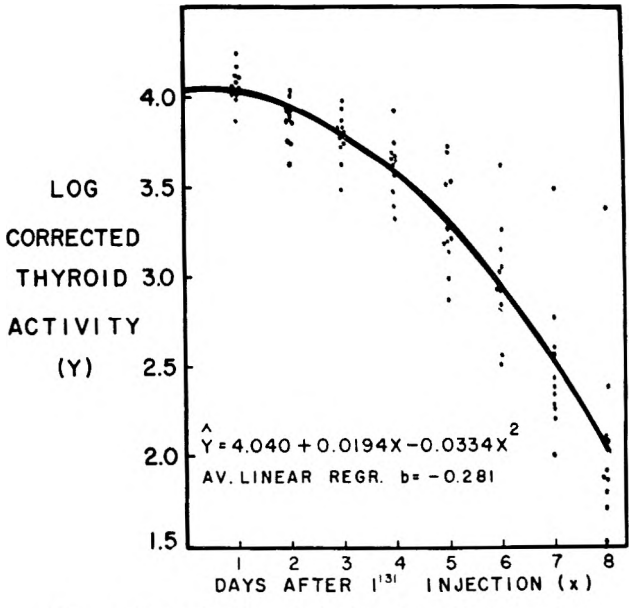

Fig. 2 Thyroid I^{131} activity in pigs 7.5 weeks of age.

about the response line. A higher order function of time was therefore computed for this secretion phenomenon. The best fitting curve was shown to be described by the following prediction equation: $\hat{Y} = 4.040 + 0.0194X - 0.0334X^2$ (X = time in days and $\hat{Y}$ = log corrected counting rate) — see figure 2. This can be explained by the hypothesis that thyrotropic hormone output would increase as the total daily thyroxine production diminished in the presence of thiouracil.

SUMMARY AND CONCLUSIONS

Thyroid function has been investigated in the young pig. It has been shown that dietary vitamin A within the range tested has considerable influence upon the rate of thyroxine secretion. Insufficient and excessive intakes of vitamin A lowered the rate of secretion. It was shown later that the relationship between this secretion rate and growth rate in the pig is rather small so that a more direct effect of vitamin A upon thyroid function is postulated.

When thiouracil was used, the rate of thyroxine secretion was shown to increase with time. An appropriate equation derived for this was: $\hat{Y} = 4.040 + 0.0194X - 0.0334X^2$ ($X =$ time after injection in days, $\hat{Y} =$ expected thyroid activity).

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PATHOLOGY OF VITAMIN B_{12} DEFICIENCY IN INFANT RATS[1]

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(Received for publication January 16, 1959)

Richardson and Hogan ('46) reported the occurrence of hydrocephalus in infant rats as the result of inadequate maternal nutrition. Because some of the vitamins were unavailable at the time, their diet was not supplemented with folic acid or vitamin B_{12}. The observations of O'Dell, Whitley and Hogan ('48) showed that a deficiency of folic acid was the chief cause of the congenital abnormality, but there is reason to believe that the diet was also somewhat limiting in vitamin B_{12}. When female rats were severely depleted of vitamin B_{12} a high proportion of the offspring were afflicted with abnormalities (O'Dell, Whitley and Hogan, '51). Hydrocephalus was the principal anomaly observed, but defects of the eyes and bones were also common. The nature of the hydrocephalus has been described (Overholser et al., '54; Newberne and O'Dell, '58).

This report is an extension of previous studies made on the central nervous system and includes observation on the peripheral nerves, lungs, kidneys and adrenal glands from vitamin B_{12}-deficient and control embryos.

[1] Contribution from Missouri Agricultural Experiment Station, Journal Series no. 1958. Approved by the Director.

[2] Public Health Service Research Fellow, National Institute of Neurological Diseases and Blindness. The data presented in this paper are taken from a thesis submitted by P. M. Newberne in the year 1958 to the Graduate School of the University of Missouri in partial fulfillment of the requirements for the Ph.D. Degree. Present address: Department of Animal Husbandry and Nutrition, Alabama Polytechnic Institute, Auburn, Alabama.

METHODS

In order to deplete the body stores of vitamin B_{12} female rats were fed, from weaning, a vitamin B_{12}-deficient diet of the following composition: soybean oil meal, 70%; glucose, 22%; lard, 4%; salts (Richardson and Hogan, '46) 4%.[3] At maturity the females were mated with normal males and the newborn observed for gross abnormalities and early mortality. The control animals were fed the basal diet supplemented with 30 μg of vitamin B_{12} per kilogram of diet. The criteria used to determine the deficiency state were a high incidence of hydrocephalus and a mortality of 80 to 90% during the first week.

For routine histological study of the newborn rats, tissues were taken from 34 hydrocephalic offspring and 35 of their litter mates which were produced by 32 vitamin B_{12}-deficient dams. There were 896 offspring born of which 92, or about 10%, were hydrocephalic. The control tissues were obtained from 28 offspring produced by 15 dams that consumed the supplemented diet. There was a total of 534 offspring born and none showed gross abnormalities. Embryos of known age were obtained by Caesarean section. Vaginal smears were made and the day sperm were observed was designated day zero in the gestation period. The brains of 15 control and 33 deficient embryos were examined at day 14; 12 control and 17 deficient at day 16 and 9 control and 18 deficient at day 18. The tissues were fixed in Bouin's or Baker's fixative, sectioned at 7 μ, and stained with either hematoxylin and eosin, gallocyanin or Herxheimer's fat stain.

A detailed study of brain tissue from embryos of different ages was made including an enumeration of the mitotic figures in the ventricular ependyma. Cells in any state of mitosis along the entire ependymal lining of the section under study were counted. A calibrated micrometer in the eyepiece of the

[3] A vitamin supplement supplied per 100 gm of diet: thiamine·HCl, 1.6; riboflavin, 1.6; pyridoxine·HCl, 1.6; Ca pantothenate, 4.0; choline·Cl, 100; biotin, 0.02; folacin, 0.5; α-tocopherol, 3.0; 2-methyl-1,4-naphthoquinone, 1.0 mg; vitamin A, 2000 I.U. and vitamin D, 280 I.U.

microscope was used to measure the area and the number of mitotic figures per unit area (0.035×0.02 mm) was calculated. Seven different portions of the ventricular system of the brain extending from the anterior lateral ventricle to the posterior cerebral aqueduct were studied. For each portion of each brain from 7 to 10 sections were counted and the number of figures per section varied from 10 to 150.

RESULTS

The cerebral parenchyma of the hydrocephalic brain was spongy and areolar with many distended spaces. The neurons were shrunken and nuclei showed vacuolation (plate 1, figs. 1 and 2). Cytoplasmic changes ranged from slight loss of Nissl substance to gross chromatolysis. The glia showed mild proliferation chiefly of oligodendroglia, some of which appeared to be concentrated about the blood vessels. The oligodendroglia and astrocytes appeared swollen and vacuolated, and in some cases exhibited degenerative changes (plate 1, figs. 3 and 4). Special stains revealed a complete lack of glycogen in the brain of experimental animals while the controls contained considerable amounts located in the choroid plexus and connective tissues.

The adrenal glands were consistently smaller in the experimental animals than in controls of the same body weight. The capsule was thin while the zona arcuata was more pronounced than normal, apparently due to deeper-staining pyknotic nuclei in the adrenal glands of the deficient animal. Many of the nuclei of the fascicular zone of the adrenal gland were pale and lacked the normal complement of chromatin. The cytoplasm of the fascicular cells of the vitamin B_{12}-deficient animals was greatly distended with lipide (plate 1, figs. 5 and 6), which gave the cell the appearance of increased cytoplasmic volume (plate 1, figs. 7 and 8). The adrenal medulla showed no significant deviation from the normal.

The lungs of the experimental offspring differed from the controls chiefly in that they typically showed delayed devel-

opment. There was poor differentiation into air spaces and the over-abundance of mesoblastic elements gave the organ a microscopic appearance of diffuse fibrosis (plate 2, figs. 9 and 10). The capillaries, for the most part, were far removed from the alveolar wall in the lung of the deficient animal (arrows).

The kidneys exhibited more retardation of growth than any other organ. The embryonic cortex consisted mainly of poorly differentiated glomeruli and tubules. The proximal tubules which had more advanced maturity were grossly dilated. Vascular extravasation and edema was notable in all areas of the deficient kidney. These observations are similar to those of Jones et al. ('55).

Although an occasional focus of poor myelination of nerve fibers in the brain of the deficient animals was observed, myelination of the central nervous system was not appreciably affected by a vitamin B_{12} deficiency. On the other hand, the spinal cord consistently showed areas without myelin and the peripheral nerves revealed either a complete absence or very little myelination of the fibers (plate 2, figs. 3 and 4). The Schwann cells of the myelin sheath appeared to be more numerous in the deficient animals, but fibrosis was not observed.

In some areas of the anterior gray columns of the spinal cord of vitamin B_{12}-deficient animals the motor neurons showed very little cytoplasmic chromatin (Nissl substance), and the nuclei in many of them were displaced or missing. Changes in the cells varied from a slight reduction in the size of Nissl bodies to complete loss of Nissl substance, the latter usually being accompanied by nuclear changes (plate 2, figs. 13 to 17). These alterations are characteristic of a cell undergoing degeneration and dissolution.

A detailed histological study of embryonic brains of rats of different ages revealed no discernible changes in the brains of 14-day embryos as a result of a maternal deficiency of vitamin B_{12}. Four of the 17 16-day deficient embryonic brains examined showed some degree of hydrocephalus and marked alterations in the aqueduct (plate 3, figs. 18 and 19). Careful

TABLE 1

Mitotic figures in the ependyma of control and vitamin B_{12}-deficient brains

Counts per unit area of ependyma (0.035 × 0.02 mm)

BRAIN LEVEL AT WHICH COUNTS WERE MADE	DAY OF GESTATION					
	Day 14		Day 16		Day 18	
	Control (15)[1]	Vitamin B_{12}-deficient (33)	Control (12)	Vitamin B_{12}-deficient (17)	Control (9)	Vitamin B_{12}-deficient (18)
Ant. Lat. Ventr.[2]	2.59 ± 0.02[3]	2.61 ± 0.04	2.76 ± 0.06	2.71 ± 0.11	0.75 ± 0.04	0.97 ± 0.04
Ant. third Ventr.	1.64 ± 0.03	1.83 ± 0.05	0.79 ± 0.04	1.04 ± 0.07	0.36 ± 0.03	0.41 ± 0.02
Mid. third Ventr.	2.43 ± 0.04	2.01 ± 0.05	0.58 ± 0.03	0.97 ± 0.04	0.66 ± 0.07	0.61 ± 0.03
Post. third Ventr.	1.82 ± 0.04	1.99 ± 0.03	0.59 ± 0.03	0.55 ± 0.04	0.96 ± 0.08	1.17 ± 0.11
Ant. Aqueduct	1.19 ± 0.02	1.48 ± 0.07	0.18 ± 0.02	0.32 ± 0.02	0.20 ± 0.03	0.45 ± 0.03
Mid. Aqueduct	1.75 ± 0.02	1.98 ± 0.05	0.13 ± 0.02	0.40 ± 0.04	0.29 ± 0.13	0.37 ± 0.02
Post. Aqueduct	1.61 ± 0.03	1.40 ± 0.04	0.33 ± 0.03	0.77 ± 0.05	0.34 ± 0.03	0.33 ± 0.02

[1] Number of brains examined.

[2] Anterior lateral ventricle.

[3] Standard error of the mean.

examination of the hydrocephalic brains suggested an increase in number of mitotic figures in the ependymal lining. In addition, there were numerous mitotic figures in the subependymal ground substance. The mitotic activity in the brains of the non-hydrocephalic litter mates was limited largely to the ependymal lining with only an occasional mitotic figure in the subependymal tissue. Three of the 18 deficient brains taken on the 18th day of gestation showed the same pattern of changes as described above. When compared to controls the mitotic figures in the deficient brain ependyma showed an increased proportion of cells in the preprophasical or early prophasical stage (plate 3, figs. 20 and 21). The evidence available indicated that an abnormal histological pattern developed sometime between the 14th and 16th day of gestation in the deficient brain.

A summary of the number of mitotic figures found in the ependyma at various levels of the brain and at different stages of embryonic development is shown in table 1. The most important finding was a larger number of mitotic figures in the aqueductal ependyma of the deficient brains on the 16th day of gestation. Statistical analysis showed that the increase observed was significant and would happen as a result of chance less than one time in one hundred.

DISCUSSION

The characteristic feature of most tissues from vitamin B_{12}-deficient embryos was their state of immaturity. The retarded development in lung, kidney and bone was particularly striking.

The failure of mesoblastic tissue of the lungs to complete the process of differentiation into normal air spaces and the lack of juxtaposition of the capillaries and air spaces would indicate reduced efficiency of gaseous exchange. The cyanosis and gasping commonly observed in the deficient animals suggests anoxia and the condition of the lungs might offer an explanation. However, when such animals were placed in an

atmosphere of high oxygen tension the symptoms were not relieved.

The retarded growth of the kidney might contribute to renal insufficiency and account for the elevated blood urea nitrogen observed by Schultze ('49) and Bruemmer, O'Dell and Hogan ('55). Dilation of the proximal tubules could have arisen from failure of the distal and proximal portions to unite in organogenesis.

The hydrocephalus which results from a vitamin B_{12} deficiency appears to be due primarily to constriction or closure of the cerebral aqueduct. Although there is a mild glial proliferation in the brains of deficient animals, it does not appear to be of sufficient magnitude to cause occlusion of the aqueduct. The greater number of mitotic figures in the aqueductal area in the 16-day embryonic brain plus the fact that the aqueduct is stenotic or occluded at this period in some of the deficient brains suggests that mitosis is not inhibited in the normal manner and continued growth of near normal brain tissue constricts the aqueduct and thus precipitates hydrocephalus.

Although an increased rate of mitosis in the aqueductal area is an attractive hypothesis to explain occlusion of the aqueduct, an increased rate of tissue growth is contrary to the delayed development observed in other organs of the vitamin B_{12}-deficient embryo. The higher proportion of mitotic figures in the ependyma of deficient brains may be the result, not of an increased, but of a decreased rate of cell division. If a metabolic block caused each cell to require a longer period to complete mitosis, there would be a piling up of mitotic figures analogous to that observed in colchicine-treated tissues. The general immaturity of tissues and the high proportion of mitotic figures in the early stages of mitosis suggest that in the vitamin B_{12}-deficient state a cell requires a longer period to assemble the materials, such as protein and nucleic acid, necessary for cell division. The observed effects of vitamin B_{12} deficiency on nucleic acid metabolism (Wong and Schweigert, '56; O'Dell and Bruemmer, '57) and on protein syn-

thesis (Wagle, Ranjan and Johnson, '58) support such a view.

It should also be noted that the number of mitotic figures per unit area in the control brains decreased rather markedly from the 14- to the 16-day embryo. Thus, if the rate of maturation were slower in the deficient embryo, its physiological age on a given day would be somewhat less than that of the control and one would expect the number of mitotic figures to be higher. From the foregoing discussion it seems unlikely that the increased number of mitotic figures in the deficient embryos is the result of increased rate of tissue growth in the mid-aqueduct area. A more reasonable explanation for the stenosis of the aqueduct in the hydrocephalic brain is a failure of the normal mitosis-arresting mechanism.

SUMMARY

A high percentage of the offspring of vitamin B_{12}-deficient rats were hydrocephalic at birth. The neurons and glial cells of the hydrocephalic brains showed irreversible degenerative changes.

The peripheral nerves were poorly myelinated whereas myelination of the brain was only slightly affected. The neurons of the spinal cord showed loss of Nissl substance along with other degenerative changes.

The adrenal glands also showed degenerative changes with accumulation of excess lipide in the fascicular zone. The lungs and kidneys were grossly immature.

There were more cells in some stage of mitosis in the ependyma of the deficient embryonic brain. The preponderance of mitotic figures in an early stage of mitosis and the general state of immaturity of the other tissues suggests that a metabolic block has slowed the rate of cell division.

ACKNOWLEDGMENT

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PLATE 1

EXPLANATION OF FIGURES

1 Neurons from the ventrolateral nucleus of the hypothalamus of a control newborn rat. Hematoxylin and eosin. × 780.

2 Neurons from an area comparable to that of figure 1 but from a vitamin B_{12}-deficient hydrocephalic brain showing edema of the tissue, and shrinkage and vacuolation of the neurons. Hematoxylin and eosin. × 780.

3 Area near cerebral aqueduct of a control newborn rat brain showing a portion of the ependymal lining at the left of the picture. Hematoxylin and eosin. × 800.

4 An area comparable to figure 3 from a vitamin B_{12}-deficient newborn hydrocephalic rat brain. The completely occluded aqueduct is at the left of the picture. Note cuboidal type ependymal cells and vacuolated nuclei of glial cells away from the aqueduct. Hematoxylin and eosin. × 800.

5 Adrenal cortex from a control newborn rat. Herxheimer's stain. × 90.

6 Adrenal cortex from a vitamin B_{12}-deficient newborn rat. Note accumulation of dark staining lipide material. Herxheimer's stain. × 90.

7 Adrenal cortex from a control newborn rat. Hematoxylin and eosin. × 750.

8 Adrenal cortex from a vitamin B_{12}-deficient newborn rat. Note pyknotic nuclei of zona arcuata at left of the photograph and lipide-laden cells of the zona fasciculata to the right. Hematoxylin and eosin. × 750.

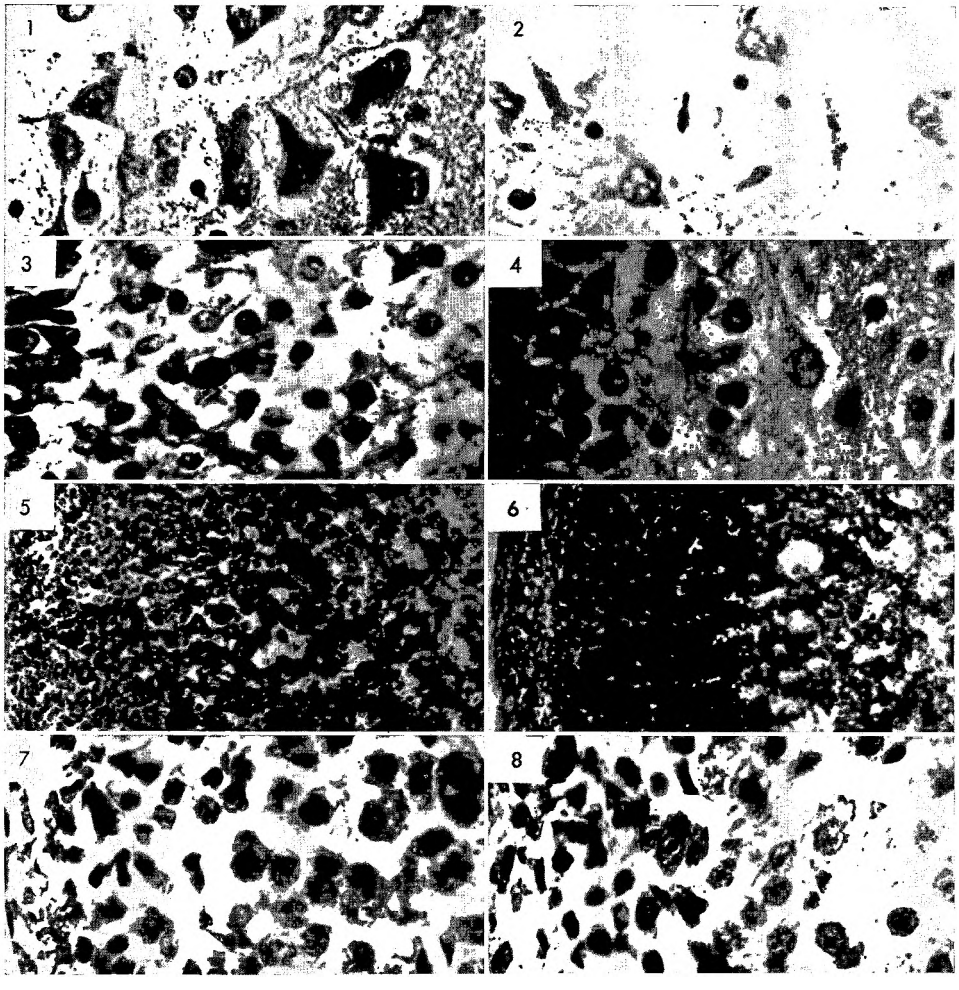
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PLATE 2

EXPLANATION OF FIGURES

9 Lung from a control newborn rat. The erythrocytes within the alveolar spaces are due to aspiration at the time of decapitation. Hematoxylin and eosin. × 800.

10 Lung from a vitamin B_{12}-deficient newborn rat. Note lack of differentiation into air spaces and the location of the capillaries (arrow) at a distance from the air space. Hematoxylin and eosin. × 800.

11 A branch of the femoral nerve of a control newborn rat showing normal myelination for this state of development. Sudan black. × 740.

12 A section comparable to figure 3 from a vitamin B_{12}-deficient newborn rat. Note complete lack of myelination of the nerve fibers. Sudan black. × 740.

13 Motor neuron from the anterior horn of the spinal cord of a control newborn rat. Gallocyanin. × 740.

14–17 Varying degrees of change in motor neurons from an area comparable to figure 5 except from a vitamin B_{12}-deficient newborn rat. Note loss of chromatin clumps (Nissl substance) and nuclear changes. Gallocyanin. × 740.

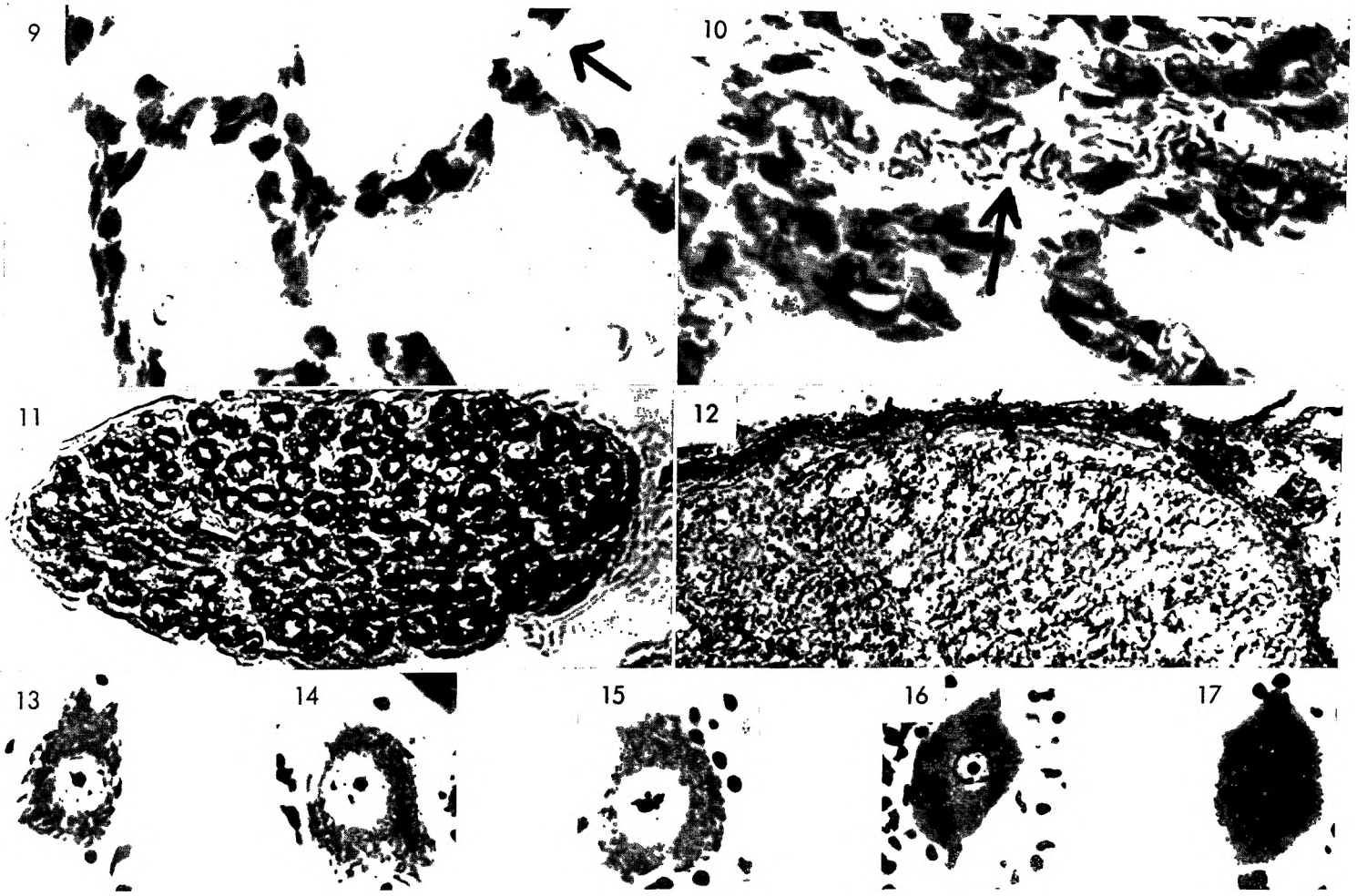
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PLATE 3

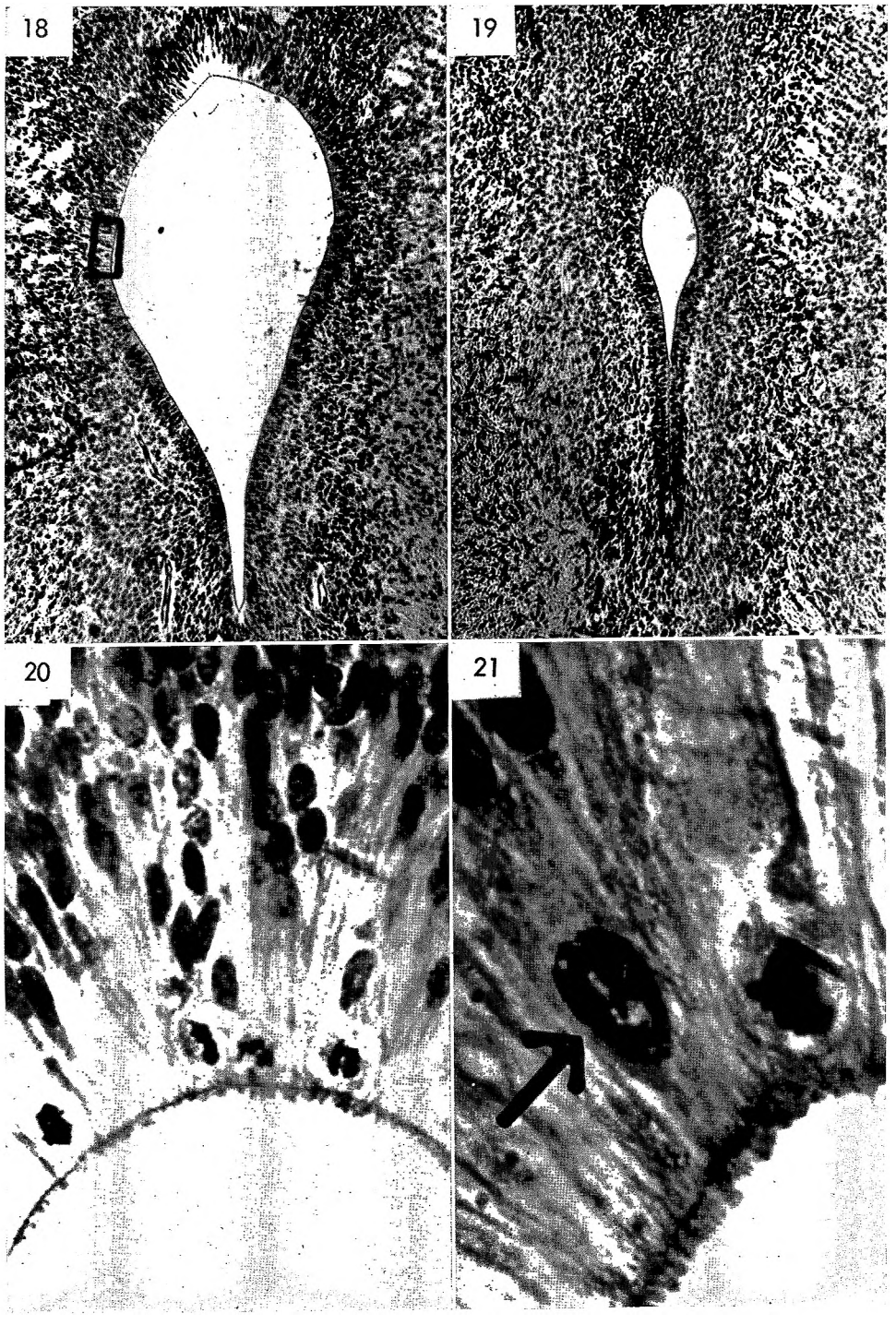
EXPLANATION OF FIGURES

18 Anterior cerebral aqueduct from a 16-day control embryo. The area marked is typical of one area in which mitotic counts were made. The mitotic figures in the entire lining ependyma were counted, the distance around the ependyma measured and the number of mitotic counts per unit area calculated. Hematoxylin and eosin. × 90.

19 Anterior cerebral aqueduct from a 16-day vitamin B_{12}-deficient embryo with hydrocephalus. Note stenosis of the aqueductal lumen and presence of tall columnar cells in the roof of the aqueduct. Hematoxylin and eosin. × 90.

20 Tall columnar cells in the roof of the cerebral aqueduct of a control 16-day embryo. Note mitotic activity. Hematoxylin and eosin. × 775.

21 Tall columnar cells in the roof of the cerebral aqueduct of vitamin B_{12}-deficient 16-day embryo. Note the cell to the left of the picture which is in the early prophase stage. There were increased numbers of cells in this stage of mitosis in the vitamin B_{12}-deficient embryonic brain. Hematoxylin and eosin. × 1600.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ALTERATIONS IN SOME BIOCHEMICAL CONSTITUENTS OF SKELETAL MUSCLE OF VITAMIN E-DEFICIENT CHICKS[1]

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Muscular degeneration is observed in chicks fed diets which are low in vitamin E and sulfur amino acids (Dam et al., '52; Machlin and Shalkop, '56), and this is prevented by including vitamin E or by increasing the levels of either methionine or cystine in the diet. Recently, selenium has been shown to be partially effective in preventing this disorder (Dam and Sondergaard, '57; Nesheim and Scott, '58). According to Dam et al. ('52), the degeneration in the skeletal muscles is accompanied by reduced creatine and increased cholesterol levels. These investigators also described the histological changes in the dystrophic chick muscle, noting particularly the perivascular infiltration with "round" cells, and stated that the changes resembled those described in muscular dystrophy from dietary causes in guinea pigs, rabbits and ducklings (Goettsch and Pappenheimer, '31; Pappenheimer and Goettsch, '34). Furthermore, studies on dystrophic muscles in man and in mice with an hereditary muscular dystrophy revealed a lowered activity of the enzyme phosphorylase (Dreyfuss et al., '54; Leonard, '57a).

This report concerns the effect of diets, with and without vitamin E, in chicks, on skeletal muscle levels of (1) glycogen and phosphorylase, (2) dry matter, ash, sodium, potassium and creatine.

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EXPERIMENTAL

Male White Plymouth Rock chicks were used in experiments 1 and 2 and equal numbers of both sexes were used in experiment 3. They were housed in thermostatically-controlled electrically-heated pens with wire-mesh floors.

The diet used in the first experiment was the torula yeast-containing diet A previously described (Nesheim and Scott, '58) except that supplementary methionine was omitted and 0.02 mg of selenium as sodium selenite was added per kilogram of the basal diet to prevent early mortality from exudative diathesis. A casein-gelatin purified diet (diet C, Nesheim and Scott, '58) was used in experiments 2 and 3. Vitamin E (*d*-α-tocopheryl acetate) was added to the control diets at the level of 160 mg/kg of diet in experiment 1, and 80 mg/kg in the others. The chicks were fed from two days of age until they were sacrificed for analysis at 5 weeks of age in experiments 1 and 2, and at 4 weeks of age in experiment 3.

The following procedure was used when samples were taken for analysis. The chicks were killed with Nembutal,[2] one-gram samples of muscle were removed immediately, frozen between two pieces of dry ice, and sealed in parafilm. These were stored at —20°C and used for phosphorylase and glycogen determinations. Separate samples were also taken for creatine and for dry weight analysis (except in experiment 3 when no dry weight determinations were made); the latter were also analyzed for ash, sodium and potassium. In experiments 1 and 2, the muscles for creatine determination were weighed, placed in a solution of 10% trichloroacetic acid and stored until analyzed. In experiment 3 the muscles were frozen and stored at —20°C until used.

The term "white" or breast muscle used in connection with determinations of phosphorylase and glycogen refers to the superficial pectoralis, and "red" or leg muscle refers to the biceps femoris. Samples taken for creatine and dry matter

[2] Abbott.

determinations included portions of both the superficial and the deep pectoralis. The appearance of the muscles was noted on autopsy and samples taken for histological studies were fixed in Bouin's solution.

Active and total phosphorylase were determined by a method previously described (Leonard, '57b) except that the amount of inorganic phosphate liberated from glucose-1-Phosphate was measured. Because of the apparently greater phosphorylase activity in chicken skeletal muscle compared to that in mammals, a reduced amount of homogenate equivalent to 2.5 mg of fresh tissue was used, and in one experiment this was further reduced to 1.25 mg. It was also observed that amounts of adenylic acid used in total phosphorylase determinations in rat muscle actually *inhibited* phosphorylase activity in the chick muscle homogenates. This difficulty was resolved by reducing the amount of adenylic acid to 0.025 mg per reaction tube, for both red and white muscle homogenates. Phosphorylase activity is reported as micrograms of inorganic P liberated by 2.5 mg (or 1.25 mg) fresh tissue from glucose-1-P in 10 minutes at 37.5°C. The ratios of active to total phosphorylase were also calculated.

The anthrone method of Seifter et al. ('50) was employed for glycogen determination. Dry weight was determined on weighed samples of muscle (about 2 gm) by heating at 105°C for 16 hours. Sodium and potassium were determined on the same samples after ashing the residue to constant weight in a muffle furnace at 550°C and extracting with 5% HCl. The extract was treated with ammonium oxalate to remove calcium before using the flame spectrophotometer for analyses. Creatine was determined by the method of Bendall ('46). In the first experiment, muscle from two chicks was combined for creatine determination but subsequently separate muscle samples were employed.

RESULTS

The average weights and incidence of muscular dystrophy in the chicks are shown in table 1. In the three experiments

nearly all the chicks fed the basal diet showed grossly visible evidence of muscle damage similar to that reported by Dam et al. ('52) and Machlin and Shalkop ('56), whereas no muscle damage was evident in chicks receiving vitamin E.

The breast muscles seemed to be affected to the greatest extent. The lesions appeared first on the breast and involved a larger proportion of the muscle fibers than in the leg muscles. The leg muscles were affected, however, since microscopic examination revealed many small areas of degenera-

TABLE 1

Body weights and incidence of muscular dystrophy in chicks used for muscle analysis and enzyme determination

TREATMENTS	AV. WT.	MUSCULAR DYSTROPHY [1]
	gm	
Experiment 1 (5 weeks)		
Basal	238	7/10 [2]
+ vitamin E	264	0/10
Experiment 2 (5 weeks)		
Basal	364	9/10
+ vitamin E	398	0/10
Experiment 3 (4 weeks)		
Basal	341	9/10
+ vitamin E	351	0/10

[1] Diagnosed by grossly visible white striations on the breast of affected chicks.
[2] Numerator is the number of chicks showing lesions, and denominator is number of chicks autopsied.

tion. Further histological examination of the dystrophic muscles showed definite degenerative changes with marked cellular infiltration, similar to that previously described.

In experiment 1, in which the torula yeast-containing diet was used, the chicks also showed symptoms of exudative diathesis. No exudative diathesis was observed in experiments 2 and 3, in which the chicks were fed the casein-gelatin diet. The muscular dystrophy observed in the chicks receiving these two experimental diets was similar in all characteristics measured.

The results of the phosphorylase and glycogen determinations are presented in table 2. A significant decrease occurred in both the active phosphorylase *a* and total phosphorylase *t* in the white muscle from vitamin E-deficient chicks, based on the wet weight of the muscle. In the first two experiments, where the lesions were more marked and the percentage of dry matter was determined, it was possible to calculate the change in phosphorylase per milligram of muscle dry weight. On this basis, the phosphorylase activity also was significantly less in the deficient chicks than in those receiving vitamin E, but the percentage difference was not as marked (table 2). For example, in experiment 1, phosphorylase *a* was 37.1% less on the wet weight basis but 27.5% less, calculated per milligram of dry weight. In red muscle, there was no significant alteration in phosphorylase activity but it was observed that both active and total phosphorylase in white muscle were twice that found in red muscle in both experimental and control groups. The ratios of active to total phosphorylase in the dystrophic and normal muscle were similar, as were the ratios in the red and white muscle. Glycogen levels were significantly reduced in the dystrophic white muscle but not in the red muscle. The glycogen levels were higher in white than in red muscle.

Measurements of dry matter, ash, sodium, potassium and creatine are presented in table 3. In both experiments 1 and 2, the muscles of chicks receiving the basal diet contained less dry matter than the muscles of chicks receiving vitamin E. The changes in potassium and creatine content of dystrophic muscles were variable; any decrease in these constituents in the dystrophic muscles could be explained mainly on the basis of increased water content. On a dry weight basis, there was little difference in the potassium and creatine content of muscles of chicks receiving the two diets. In experiment 3 the creatine was significantly lower in the dystrophic muscles, as expressed on wet weight basis only.

The sodium content of the breast muscles of chicks receiving the basal diet was significantly higher on both wet and

TABLE 2

Phosphorylase in muscles from chicks receiving a vitamin E-deficient diet

MUSCLE AND EXPERIMENT	DEFICIENT				CONTROL			
	P-lase *a* [1]	P-lase *t* [2]	*a*/*t* ratio × 100	Glycogen	P-lase *a*	P-lase *t*	*a*/*t* ratio × 100	Glycogen
	μg P	*μg P*		*mg%*	*μg P*	*μg P*		*mg%*
				Wet weight basis				
White muscle, exp. 1	*222* ± 21.5 [3]	—	—	*511* ± 41 [4]	353 ± 14.4	—	—	844 ± 46.7
White muscle, exp. 2	*274* ± 17.2	*330* ± 19.5	83.4 ± 1.5	*670* ± 42.8	378 ± 13.2	447 ± 18.5	84.6 ± 0.6	901 ± 27.0
Red muscle, exp. 2	146 ± 9.0	166 ± 11.4	88.1 ± 1.3	534 ± 43.8	178 ± 14.6	207 ± 15.0	85.6 ± 1.5	441 ± 29.7
White muscle,[5] exp. 3	*167* ± 11.7 [6]	*194* ± 13.9	86.1 ± 1.4	*787* ± 33.8	200 ± 4.1	231 ± 4.5	86.3 ± 1.1	1074 ± 31.6
Red muscle, exp. 3	160 ± 17.6	186 ± 19.7	85.5 ± 0.9	512 ± 55.0	118 ± 13.7	135 ± 13.5	87.0 ± 1.2	506 ± 30.5
				Dry weight basis				
	μg P/mg DM	*μg P/mg DM*		*mg/gm DM*	*μg P/mg DM*	*μg P/mg DM*		*mg/gm DM*
White muscle, exp. 1	*440* ± 36.1	—		*24.7* ± 1.8	608 ± 27.6	—		36.7 ± 2.1
White muscle, exp. 2	*467* ± 25.6	*561* ± 31.5		*28.5* ± 1.6	597 ± 23.5	709 ± 32.2		35.6 ± 1.0

[1] Active phosphorylase.
[2] Total phosphorylase.
[3] Figures in italics indicate significant difference from controls ($P < 0.01$).
[4] Only 8 determinations (2 lost), all the rest 10 each.
[5] P-lase activity per 1.25 mg of muscle, all others 2.5 mg of muscle.
[6] In this instance, $P < 0.02$.

TABLE 3

Dry matter, ash, sodium, potassium and creatine content of breast muscles

TREATMENT	PER CENT DRY MATTER	PER CENT ASH OF DRY MATTER	SODIUM, MG/100 GM		POTASSIUM, MG/100 GM		CREATINE, MG/GM	
			Fresh muscle	Dry matter	Fresh muscle	Dry matter	Fresh muscle	Dry matter
Experiment 1								
Basal diet	(10)[1] *20.1*[2] ± 0.46	5.66 ± 0.27	(8) *112* ± 12	*553*[3] ± 82	(8) *263* ± 24	1316 ± 89	(5) 5.6[4] ± 0.30	27.8 ± 1.0
+ vitamin E	(10) 23.3 ± 0.22	5.30 ± 0.14	(9) 74 ± 3	317 ± 17	(9) 344 ± 12	1482 ± 49	(5) 5.9 ± 0.42	25.6 ± 1.8
Experiment 2								
Basal diet	(9) *23.4* ± 0.50	6.99 ± 0.36	(6) *99* ± 11	*415* ± 54	(6) 285 ± 19	1188 ± 77	(8) *5.3* ± 0.26	23.0 ± 0.58
+ vitamin E	(9) 25.3 ± 0.39	5.98 ± 0.40	(9) 65 ± 5	255 ± 14	(9) 316 ± 7	1252 ± 42	(10) 5.9 ± 0.13	23.3 ± 0.58
Experiment 3								
Basal diet							(8) *4.7* ± 0.23	
+ vitamin E							(9) 5.6 ± 0.20	

[1] Figures within parentheses indicate number of determinations.

[2] Figures in italics indicate significant difference from the controls ($P < 0.01$).

[3] In this instance, $P < 0.025$.

[4] In this experiment creatine determined on pooled muscle sample from two chicks, other experiments individual chicks used.

dry weight basis than that of the supplemented group. The ash content of the muscles of the vitamin E-deficient chicks was higher than in the controls, but the increase was not significant statistically.

DISCUSSION

The chick is a useful experimental animal in which to study the muscular dystrophy caused by vitamin E deficiency. The lesions develop in about three to 4 weeks on the experimental diets, and are easily observed as grossly visible white striations in the breast muscles. The chicks showing extensive damage to the breast muscle presented no indication of this in their outward appearance. As opposed to the findings in mammals, no weakness of the limbs was observed. The body weight gains of the chicks deficient in vitamin E were only slightly below those of the controls.

In contrast to what is generally observed in other animals (Goettsch and Brown, '32; Blaxter and McGill, '55), the creatine content of the muscle was reduced very little even when expressed on a wet muscle basis, and on a dry basis there was no change at all.

The position of phosphorylase in the synthesis and degradation of muscle glycogen warrants a study of this enzyme in conditions of muscular dystrophy. The evidence presented indicates that both active and total phosphorylase are significantly depressed in the white breast muscle of the vitamin E-deficient chick. The results in the last experiment were not as striking as those of the first two experiments. This may have been due to the fact that the birds were autopsied too soon. Since ordinary histological examination of the muscles showed that all fibers were not injured, the question arises as to whether the decreased phosphorylase was due to alterations in the protein of these injured fibers, which was reflected in the over-all decrease in enzyme activity, or were all the fibers affected with biochemical lesions of varying degrees of severity.

The level of phosphorylase activity was found to be considerably lower in red muscle than in white. Although definite microscopic lesions were present in the vitamin E-deficient red muscles, little gross muscle damage occurred, and no consistent changes in phosphorylase activity were detected. In studies of muscular dystrophy in the rabbit, Goettsch and Brown ('32) reported a greater severity of lesions in white muscle than in red.

The activity ratios of phosphorylase were similar in normal and dystrophic muscles. Thus, while degenerating muscle loses its total phosphorylase, the phosphorylase *a* remains proportionately constant. A similar situation prevails in skeletal muscle of mice, with hereditary muscular dystrophy (Leonard, '57a). Other studies indicate that changes in phosphorylase *a*, which account for ratio alterations, can be brought about by sufficient contraction of muscle. In this case, the total phosphorylase remains the same but phosphorylase *a* is considerably reduced. Adrenalin will maintain the level of phosphorylase *a* during contractions in rat skeletal muscle (Leonard, '57b) but not in uterine smooth muscle (Leonard, '58).

It was also noted that adenylic acid requirements to demonstrate total muscle phosphorylase are much less in the chick than in the mammal. In the chick studies, an excess of adenylic acid in the reaction tube not only failed to permit measurement of total enzyme but actually inhibited the activity of phosphorylase *a* present.

The glycogen levels of the dystrophic breast muscles were also lower than in the controls but no difference was noted in the red muscles even though lesions were present. White breast muscle has basically a higher level of glycogen than red muscle. While it might seem logical to relate the changes in phosphoryase to glycogen levels, this situation does not obtain in the hereditary dystrophic mice. In the latter, changes in phosphorylase parallel those found in the vitamin E-deficient chicks, yet the concentration of glycogen in the dystrophic mouse muscles was higher than in the controls.

Dystrophic changes in skeletal muscle from different causes will probably have different patterns of alteration in their biochemical constituents.

SUMMARY

1. Chicks were fed two different muscular dystrophy-producing diets, with and without vitamin E, and determinations of glycogen, phosphorylase, dry matter, ash, sodium, potassium and creatine were made on the breast (white) and leg (red) muscles after 4 to 5 weeks of feeding.

2. Body weights were approximately the same and the birds showed no outward manifestation of muscle dystrophy just before autopsy.

3. Gross lesions in the dystrophic muscle were more apparent in the white than in the red muscle, although microscopic detection of lesions in the latter was possible.

4. The changes in creatine and potassium content of dystrophic muscles were variable and any decrease in these constituents in the dystrophic muscles could be explained mainly on the basis of increased water content. The sodium content of dystrophic muscles was increased when expressed on either a wet or dry weight basis.

5. The percentage of dry matter was less in the deficient chick breast muscle than in the normal.

6. Active phosphorylase *a* and total phosphorylase *t* concentrations on both wet and dry weight basis were lower in the white muscle of the deficient chick but no consistent changes were detected in red muscle. Although both phosphorylase measurements were higher in white than in red muscle under all conditions, the ratios of a/t were always the same.

7. Glycogen levels were lower in the white dystrophic muscles in comparison with the controls but no difference was observed between the normal and dystrophic red muscle. White muscle is basically richer in glycogen than red muscle.

8. Both diets gave essentially the same results and 5 weeks is a preferred period for these changes to develop.

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THE EFFECT OF DIETARY FAT ON VITAMIN B_{12}-METHIONINE INTERRELATIONSHIPS [1]

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It was previously found that raising the fat level of a corn-soybean meal diet from 3 to 22% increased the severity of a vitamin B_{12} deficiency in non-depleted chicks (Spivey, Briggs and Ortiz, '54) and elevated the vitamin B_{12} requirement (Fox, Ortiz and Briggs, '56). This high vitamin B_{12} requirement could be eliminated by supplemental methionine (Fox, Briggs and Ortiz, '57). The usefulness of this crude diet for vitamin B_{12} studies was limited since it did not permit the omission or variation in level of most nutrients. In the present paper are reported studies undertaken to duplicate these dietary effects using only purified constituents. A satisfactory purified diet was developed and the vitamin B_{12}-sparing effect of methionine has been studied in chicks fed this diet formulated to contain 0, 4 and 24% fat.

EXPERIMENTAL PROCEDURE

Female New Hampshire chicks were distributed into groups of 6 chicks each at one day of age. They were maintained in electrically heated batteries with screen floors, received diet and water ad libitum, and were weighed at weekly intervals. The experiments were terminated at the end of 4 weeks.

[1] A preliminary report on some of these data was given at the annual meeting of the American Institute of Nutrition, Philadelphia, April 14–18, 1958.

Diet C47, which was the basis for all diets used in these experiments, had the following composition (grams per kilogram of diet): soybean protein [2] 300, L-cystine 3, corn oil 40, salts A (Briggs et al., '52) 60, vitamins 1, and crude glucose [3] 596. The following vitamins were added (milligrams per kilogram of diet): thiamine·HCl 8, riboflavin 8, calcium pantothenate 20, choline chloride 1000, nicotinic acid 100, pyridoxine·HCl 8, *d*-biotin 0.3, pteroylglutamic acid 3, vitamin A acetate 6, vitamin D_3 0.02, α-tocopherol (free) 25, α-tocopherol acetate 25, and 2-methyl-1,4-napthoquinone 1. When present, vitamin B_{12} was added at a level of 0.1 mg per kg of diet. This diet was modified to contain a total of 24% fat by replacing 200 gm of glucose with 200 gm of hydrogenated vegetable oil [4] per kg of diet. Diet C47 was also modified, as indicated in the tables, so that all fat was omitted. For convenience, this latter diet is referred to as containing 0% fat; actually a small amount of fat (less than 0.15% of the diet, according to the manufacturer's analysis) was present in the soybean protein. In the fat-free diets, vitamin D_3 and 2-methyl-1,4-naphthoquinone were added to the diet in alcoholic solutions. Vitamin A acetate was administered in the drinking water as suggested by Bieri ('57). Vitamins E and K were also added by a similar procedure [5] to the drinking water of chicks fed fat-free diets. Supplements of DL-methionine ranging from 0.1 to 1.5% of the diet were added to diets containing each level of fat. Dietary supplements were made at the expense of an equal weight of glucose. The effects of fat

[2] Drackett Assay Protein Cl, purchased from Archer-Daniels-Midland Company, Cincinnati, Ohio.

[3] Cerelose.

[4] Crisco.

[5] Two-hundred milligrams of α-tocopherol (free) were dissolved in 1 ml ethanol and mixed with 2 ml Tween 80 and 7 ml water. This mixture was added to a liter of drinking water. Ten milligrams of menadione sodium bisulfite (Abbott Laboratories, Chicago) were dissolved in 5 ml water and added per liter of water. Both mixtures were prepared in quantities larger than the above and stored in the refrigerator for as long as a month. Both vitamins were administered twice weekly.

level and methionine supplementation were studied in both the absence and presence of dietary vitamin B_{12}.

At the end of most experiments the chicks were fasted for 18 hours and were bled from the heart. The serum was analyzed for total lipid by the method of Bragdon ('51). Phospholipid was estimated by determination of phosphorus by the method of Fiske and Subbarow as outlined by Umbreit, Burris and Stauffer ('49). Total cholesterol was determined by the method of Zak et al. ('54).

RESULTS

The effects of methionine upon growth of vitamin B_{12}-deficient and supplemented chicks fed diets containing 0, 4, and 24% of fat are presented in table 1. In the groups that re-

TABLE 1

Effect of dietary fat and methionine on the mortality and growth of chicks deficient and supplemented with vitamin B_{12}

METHIONINE SUPPLEMENT	NO VITAMIN B_{12}		100 μG VITAMIN B_{12}/KG DIET	
	Mortality No. chicks [1]	Mean 4-week weight and S.E.	Mortality No. chicks [1]	Mean 4-week weight and S.E.
%		*gm*		*gm*
No dietary fat				
None	1/24	240 ± 11	1/24	309 ± 9
0.3	4/18	245 ± 14	3/18	284 ± 15
1.0	0/12	190 ± 12	2/12	223 ± 17
4% dietary fat				
None	3/42	229 ± 6	1/42	326 ± 30
0.1	0/6	251 ± 25	0/6	326 ± 19
0.3	1/30	359 ± 8	1/30	312 ± 42
1.0	2/18	338 ± 12	1/18	383 ± 12
1.5	0/6	239 ± 19	1/6	306 ± 27
24% dietary fat				
None	3/24	150 ± 9	1/24	302 ± 10
0.1	1/12	244 ± 12	0/12	328 ± 12
0.3	0/12	320 ± 13	0/12	365 ± 13
1.0	1/12	324 ± 16	0/12	381 ± 15
1.5	0/6	263 ± 17	0/6	311 ± 18

[1] Values for each dietary treatment are averages of one to 7 experiments, as indicated by the total number of chicks (6 chicks per experimental group).

ceived no vitamin B_{12} and no supplemental methionine, mean weights at 4 weeks decreased as the fat content of the diet was increased. When vitamin B_{12} was added to these same diets, however, growth was equally good with either 0, 4 or 24% of fat in the diet. When the several experiments are considered together, as they are averaged in table 1, the omission of vitamin B_{12} from the diet resulted in a significant depression of growth with each level of fat. The growth depression was also statistically significant in each individual experiment for the chicks receiving diets containing either 4 or 24% of fat. With no added fat in the diet, however, a statistically significant difference between the vitamin B_{12}-deficient and the vitamin B_{12}-supplemented groups was seen in only one out of 4 experiments.

With 4% of fat in the diet, the addition of methionine at 0.3 and 1% raised the growth rate of vitamin B_{12}-deficient chicks to equal that obtained in chicks receiving vitamin B_{12}. The same effect was observed with 24% of fat in the diet. The 1.5% level of methionine was toxic in both fat-containing diets irrespective of vitamin B_{12} status. Also, with both diets, 1% of methionine plus vitamin B_{12} improved the growth rate over that obtained with the vitamin alone; thus methionine could not completely replace vitamin B_{12}.

Of special interest is the effect of supplemental methionine in the absence of fat. Methionine did not replace or spare vitamin B_{12}; in fact, the 1% level of methionine had an adverse effect upon feathering, leg bone formation, and growth. As shown in figure 1, the wing feathers appeared ragged since they broke very easily, pigmentation was often deficient or lacking, and feathering across the back, breast, and on the legs was retarded. Perosis and the severely defective feathering were seen in about one-fourth to one-half of the chicks fed the vitamin B_{12}-deficient, fat-free diet containing 1% of methionine. Vitamin B_{12} partially protected against these defects.

The effect of dietary fat level, methionine supplements, and vitamin B_{12} upon food and energy intake may be seen in

Fig. 1 Defective feather formation in a chick fed a diet free of vitamin B_{12} and fat, supplemented with methionine at the 1% level.

table 2. Vitamin B_{12}-deficient chicks unsupplemented with methionine utilized the diet with equally poor efficiency at each level of fat intake. As a result, the chicks receiving 24% of dietary fat had a considerably higher caloric intake per unit gain. The chicks receiving vitamin B_{12} utilized the diet

TABLE 2

Effect of vitamin B_{12}, fat and methionine upon intake of food[1] and calories during four-week experiment

NO. EXPERIMENT	DIETARY FAT	METHIONINE SUPPLEMENT	NO VITAMIN B_{12}			100 μG VITAMIN B_{12}/KG DIET		
			Mean weight gain	Food intake	Caloric intake	Mean weight gain	Food intake	Caloric intake
	%	%	*gm*	*gm/gm gain*	*Cal./gm gain*	*gm*	*gm/gm gain*	*Cal./gm gain*
4	0	0	201	2.33	8.48	270	1.92	6.99
7	4	0	189	2.33	8.48	285	1.92	6.99
3	24	0	110	2.35	11.07	262	1.69	7.96
2	0	1	152	2.43	8.33	185	2.13	7.30
3	4	1	300	1.75	6.37	345	1.58	5.75
2	24	1	285	1.52	7.16	342	1.51	7.11

[1] Except when the chicks received a methionine supplement, the same relative differences would apply to the intake of methionine and protein as to food intake since the percentage of protein in the diet remained constant.

TABLE 3

Effect of dietary fat and methionine on serum lipid constituents of chicks deficient and supplemented with vitamin B_{12} [1]

METHIONINE SUPPLEMENT	NO VITAMIN B_{12}			100 μG VITAMIN B_{12}/KG DIET		
	Total fat	Phospholipid	Total cholesterol	Total fat	Phospholipid	Total cholesterol
%	*mg%*	*mg%*	*mg%*	*mg%*	*mg%*	*mg%*
No dietary fat						
None	714 ± 29	271 ± 20	133 ± 6	708 ± 33	276 ± 22	139 ± 6
1	767 ± 33	327 ± 20	153 ± 12	695 ± 44	265 ± 21	135 ± 7
4% dietary fat						
None	799 ± 16	288 ± 12	162 ± 3	736 ± 30	268 ± 11	158 ± 8
1	847 ± 30	310 ± 18	171 ± 10	726 ± 42	267 ± 16	158 ± 7
24% dietary fat						
None	781 ± 48	271 ± 24	160 ± 9	814 ± 40	287 ± 21	189 ± 12
1	814 ± 19	309 ± 15	199 ± 10	722 ± 55	263 ± 13	160 ± 10

[1] Mean values from two experiments ± S. E.

efficiently at each level of fat intake and the caloric intake remained relatively constant. The beneficial effects of methionine upon growth were also reflected in more efficient utilization of the diets containing added fat.

In table 3 are presented mean concentrations of total fat, phospholipid, and total cholesterol in the serum of chicks from some of the groups described above. Considerable variability within groups was observed, especially in the values for total fat and phospholipids. No clear-cut effect upon the concentration of any of these lipid components could be associated with any of the dietary changes. Serum lipid values for chicks receiving other dietary levels of methionine were unaffected by the fat or vitamin B_{12} content of the diet; therefore, these data were not included in table 3.

DISCUSSION

The usefulness of purified diet C47 for studying interrelationships between vitamin B_{12} and other nutrients is important. Since this diet is composed of highly purified ingredients, the level of any nutrient may be varied. Also, with this diet it is not necessary to use chicks from hens that had been on a vitamin B_{12}-deficient ration in order to produce a deficiency of the vitamin.

The use of the purified soybean protein diet in studies of the relationships between dietary fat and severity of vitamin B_{12} deficiency has confirmed the earlier work with the corn-soybean meal diets (Spivey, Briggs and Ortiz, '54). The mechanism whereby fat increased the vitamin B_{12} deficiency and by which supplemental methionine partially replaced the vitamin is not known. Certain aspects of these interrelationships are, however, clearly defined. From the earlier studies with the crude diet, it was shown that neither high-fat intake nor supplemental methionine with high dietary fat affected the storage of vitamin B_{12} in the chick's liver (Fox, Ortiz and Briggs, '56; Fox, Briggs and Ortiz, '57). Also, with the high-fat corn-soybean meal diet, neither choline (Fox, Briggs and Ortiz,

'57), betaine, nor homocystine spared vitamin B_{12}.[6] The combination of choline and homocystine was equal to methionine in replacing the vitamin. It is not likely that the increased need for vitamin B_{12} with the high-fat diet depends upon methyl group transfer from choline to homocysteine. The important relationship between vitamin B_{12} and methionine may involve synthesis or transfer of a methyl group from some source other than choline or betaine, or both.

The methionine level of the diet was critical in the development of a vitamin B_{12} deficiency, as shown in these studies. Based on calculated amino acid content, diet C47 is borderline in the sulfur-containing amino acids. It was found that if the 0.3% of L-cystine in the diet was replaced with methionine, no vitamin B_{12} deficiency was produced.[6] As the fat content of the diet was increased, the caloric density of the diet also increased; therefore, food consumption to meet energy needs alone would be expected to result in exaggeration of this borderline methionine deficiency.

The data on food and energy intake of vitamin B_{12}-deficient chicks seem to indicate an inability to utilize high levels of dietary fat. This suggests a possible role for vitamin B_{12} or methionine or both in the metabolism of fat. Supplementary data on absorption of dietary fat and on carcass composition might help to establish the factors involved. In the presence of vitamin B_{12}, food intake was lowered as the fat content of the diet increased. If vitamin B_{12} does function in protein synthesis, as proposed by Wagle and co-workers ('58), then the effect of vitamin B_{12} in the high-fat diets could be attributed to more efficient, but non-specific, utilization of the available methionine.

The marked increase in toxicity of methionine in the fat-free diets may also be related to a possible role for methionine in the utilization of fat. Evidence for such a role has been obtained from studies in rats receiving toxic levels of methionine (Roth, '57). Since the chick is slow to develop an

[6] Unpublished data.

essential fatty acid deficiency (Bieri et al., '56), essential fatty acids were thought not to be involved in the present study.

The serum lipid levels indicated no gross abnormality in fat metabolism. The livers appeared normal and had normal concentrations of total lipids and phospholipids.[6] If derangement in fat utilization is involved in producing a vitamin B_{12} deficiency in chicks fed the high-fat diet, certainly more sensitive parameters of response are required to detect it.

Considerable variability has occurred among experiments in the growth of chicks fed the 4% fat diet containing vitamin B_{12}, as reflected in the large standard error (table 1). The same variability was observed with 0.3% of methionine in the diet. In some experiments in which growth of this control group was low, an improvement in growth was obtained with a supplement of choline at 0.05% of the diet.[6] It is possible that a borderline deficiency of choline was present in some groups (due to variable stores in the chicks and contamination of dietary protein); however, supplementary choline did not stimulate growth of chicks fed the high-fat diet containing vitamin B_{12}.

SUMMARY

A purified diet containing isolated soybean protein was developed for the study of vitamin B_{12} in the non-depleted chick. The vitamin B_{12}-sparing effect of methionine in day-old New Hampshire chicks receiving 0, 4 and 24% fat in the diet was determined with the new diet. In confirmation of earlier studies with a crude corn-soybean meal diet, increasing the fat content of the diet increased the severity of the vitamin B_{12} deficiency that was obtained during 4-week experimental periods. This effect of high dietary fat was lost in the presence of supplemental methionine; however, both vitamin B_{12} and methionine were necessary for maximum growth. With no fat in the diet, growth depression in the absence of vitamin B_{12} was very small. In the absence of fat

[6] See footnote 6, page 379.

the toxicity of methionine was markedly increased, as indicated by poor growth, poor feather formation, and a moderate incidence of perosis. Some of these changes were less severe in vitamin B_{12}-supplemented chicks. Serum concentrations of total fat, phospholipids, and total cholesterol were not markedly affected by vitamin B_{12} deficiency, fat level of the diet, or methionine supplementation. Possible roles of vitamin B_{12} and methionine in fat metabolism and protein synthesis were discussed in relation to these findings.

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A COMPARISON OF THE NUTRITIVE VALUE OF ALFALFA HAY WITH BROME GRASS AND REED CANARY GRASS HAYS AT VARIOUS LEVELS OF NITROGEN FERTILIZATION [1]

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The total digestible nutrients (TDN) method of determining the nutritive value of feedstuffs for livestock has repeatedly been criticized because the several procedures required by the method make it a cumbersome and laborious one (Lofgreen, '51; Maynard, '53; and Swift, '57). In recent years the digestible energy (DE) method of evaluation has received renewed interest (Crampton et al., '57; Lofgreen, '57), since it measures the same availability of energy to the animal as does TDN. The apparent advantage of DE is that the energy content is obtained with ease and accuracy from one determination of the feed and the feces. Swift ('57) states that the relationship between both systems is expected to be high and believes that any deviation from perfect cor-

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Animal Husbandry, New Brunswick. This study was part of a Northeast Regional Project, NE-24, "The Nutritive Evaluation of Forages," a cooperative study involving agricultural experiment stations in the Northeastern Region, the Dairy Husbandry Research Branch, USDA, and the U. S. Regional Pasture Research Laboratory and supported in part by regional funds.

[2] Part of the data presented herein were taken from a thesis presented by K. M. Barth to the graduate faculty of Rutgers University in partial fulfillment of the requirements for the degree of Master of Science.

relation would be due to the inherent errors in the TDN method. Correlation coefficients of + 0.97, + 0.94, and + 0.86 between the DE and TDN methods of evaluation have been reported by Swift ('57), Macdonald ('57), and Markley ('58), respectively, from trials conducted with various feeds and species of livestock.

Increased yields with increasing nitrogen fertilization on brome grass were reported by Anderson et al. ('46), and Russell et al. ('54). Laughlin ('53) and Wagner ('54) reported both increased yield and increased protein content with higher nitrogen applications on brome grass. Ramage ('56) reported similar responses on reed canary grass. Higher nitrogen applications have also caused an increase in the apparent digestibility of protein (Ferguson, '48; Moon, '54; Poulton et al., '57; Markley, '58). It was also reported by Raven and Robinson ('57) that forages grown with higher levels of nitrogen application effected increased protein and gross energy content with increases in the digestibility of protein, ether extract, and fiber, while the digestibility of nitrogen-free extract (NFE) decreased.

The objectives of the investigation were: (1) to determine the relationship between TDN and DE; (2) to determine the effect of the application of various levels of nitrogen on brome grass and reed canary grass on the digestion coefficient of protein, ether extract, crude fiber, and energy, and to compare these digestibilities with those of alfalfa hay, grown with conventional fertilizer application.

MATERIALS AND METHODS

Brome grass trial ('56). To obtain hays of various nutritive values, pure stands of brome and reed canary grass were treated with various levels of nitrogen per acre, and used as experimental hays for the determination of the TDN and DE relationship.

Four Western wether lambs of similar weight (averaging 70 pounds), breeding, and conformation were selected and

used in this study. After being wormed with phenothiazine and shorn they were randomly assigned to metabolism crates, as described by Bratzler ('51), and the following hays were fed according to a standard 4 x 4 Latin square design, the alfalfa hay having been harvested at the half bloom stage and the brome grass hays, during the headed to early bloom stage:

Alfalfa hay, second cutting (harvested July 18th), grown with 600 pounds of 0–5–30[3] per acre;

Brome grass hay (low), second cutting (harvested June 14th), grown with 25 pounds of nitrogen per acre;

Brome grass hay (medium), second cutting (harvested June 14th), grown with 125 pounds of nitrogen per an acre; and,

Brome grass hay (high), second cutting (harvested June 14th), grown with 225 pounds of nitrogen per acre.

Each 7-day collection period was preceded by a 10-day preliminary period. During both periods the hays were fed in equal amounts and refused feeds were kept at a minimum. For each collection period, daily hay samples were taken along with any "weighback" samples that may have been present. Samples of feces and urine were taken daily from each animal, and at the end of 7 days, these samples were composited. This composited sample of feces or urine was blended and a sample was drawn for proximate analysis. The proximate analysis of protein, ether extract and fiber was determined according to procedures outlined by the A.O.A.C. ('55), while energy determinations were made in an Emerson adiabatic-type fuel calorimeter.

Reed canary grass trial ('57). For the second digestion trial, 4 uniform Hampshire wether lambs (averaging 90 pounds) were selected, randomly assigned to one of the following rations, and fed according to the experimental regime outlined for the brome grass trial. The alfalfa hay was harvested at the early bloom stage; the first cutting of reed canary grass

[3] Fertilizer mixture applied to hay crop containing 0% (N), 5% (P205) and 30% (K).

hay, in the early boot stage; and the second cuttings of reed canary grass hay, at a comparative uniform height:

Alfalfa hay, second cutting, grown with 600 pounds of 0–10–20 [4] per acre; reed canary grass hay (low), first cutting (harvested June 8th), grown with no nitrogen fertilization, pure stand;

Reed canary grass hay (medium), second cutting (harvested July 22nd), grown with 100 pounds of nitrogen per acre, estimated to contain 30% orchard grass, rained on (0.09 inches); and,

Reed canary grass hay (high), second cutting (harvested July 23rd), grown with 200 pounds of nitrogen per acre, estimated to contain 15% orchard grass.

RESULTS AND DISCUSSION

Analytical data obtained in this study are presented in table 1. They include average chemical composition of the brome grass, alfalfa hay and reed canary grass with respect to moisture, ash, fiber and the energy-yielding nutrients with their digestion coefficient factors; in addition data are given on total digestible nutrients (TDN), digestible dry matter and nitrogen retention.

Brome grass trial ('56). It was observed that as the level of nitrogen fertilization increased, a corresponding increase in the protein content of the brome grass hays occurred. At the highest level of nitrogen application, the protein content exceeded that of alfalfa. Within the limits of this study, the effect of nitrogen fertilization on the protein content of brome grass hays approached linearity. There were also small decreases in fiber and nitrogen-free extract, and increases in gross energy and ether extract with increasing nitrogen fertilization.

Increased nitrogen fertilization on brome grass resulted not only in an increase in the protein content, but also an increased

[4] Fertilizer mixture applied to hay crop containing 0% (N), 10% (P205) and 20% (K).

TABLE 1

Average composition, digestion coefficients of nutrients, digestible nutrients, digestible energy, total digestible nutrients, nitrogen retention, and digestible dry matter of alfalfa hay and high nitrogen grass hays. (Air-dry basis)

	BROME GRASS TRIAL				REED CANARY GRASS TRIAL			
	Alfalfa hay	Brome grass hay			Alfalfa hay	Reed canary grass hay		
		25 lb. N/A [1]	125 lb. N/A	225 lb. N/A		0 lb. N/A	100 lb. N/A	200 lb. N/A
Protein:								
Crude, %	14.83	7.94	11.34	15.23	15.39	12.55	14.55	20.05
Dig. coeff., %	71.23	61.00	66.22	73.30	74.63	68.48	70.43	77.67
Digestible, %	10.56	4.84	7.51	11.16	11.49	8.59	10.25	15.57
Ether Extract:								
Crude, %	2.15	2.10	2.57	2.63	2.14	3.16	3.47	3.58
Dig. coeff., %	41.28	35.30	40.18	46.78	14.46	47.28	30.14	39.45
Digestible, %	0.89	0.74	1.03	1.23	0.31	1.49	1.05	1.41
Fiber:								
Crude, %	27.23	31.63	29.43	27.86	23.05	23.37	23.67	20.39
Dig. coeff., %	40.58	60.69	63.31	61.30	41.36	63.51	59.04	62.60
Digestible, %	11.05	19.20	18.63	17.08	9.53	14.84	13.97	12.76
N-free extract:								
Crude, %	39.76	43.91	42.92	39.37	41.16	44.36	40.12	36.84
Dig. coeff., %	65.43	61.00	62.55	61.07	75.48	69.99	62.64	64.04
Digestible, %	26.01	26.79	26.85	24.04	31.07	31.05	25.13	23.59
Ash, %	6.27	6.49	5.20	5.33	7.30	6.20	7.03	6.70
Moisture, %	9.76	7.93	8.54	9.59	10.96	10.36	11.16	12.44
Energy:								
Gross, Cal./gm	4,216	4,159	4,264	4,285	4,118	4,158	4,245	4,224
Dig. coeff., %	55.74	57.41	59.75	60.59	60.33	63.18	58.08	62.36
Digestible, Cal./gm	2,350	2,388	2,548	2,596	2,484	2,627	2,465	2,634
TDN, %	49.70	52.57	55.37	55.17	52.92	57.94	51.75	55.43
Nitrogen retention (gm/7 days)	32.41	10.98	21.39	37.22	32.59	28.23	29.77	22.26
Digestible dry matter, %	57.11	59.79	62.14	62.67	62.67	66.37	61.48	65.44

[1] Twenty-five pounds of nitrogen per acre.

protein digestibility. At the highest level of fertilization, the protein digestibility of brome grass was even higher than that of alfalfa hay. Application of the multiple range test (Duncan, '55) showed that the differences were statistically significant ($P < 0.05$). There was no appreciable difference between the fiber digestion coefficient of the brome grass hays, but the fiber digestibility of alfalfa hay was consistently 20 percentage points lower than that of all the brome grass hays. The differences observed in the digestibilities of nitrogen-free extract and ether extract components were not significant, while the increase of energy digestibility was significantly higher ($P < 0.05$) for the high brome grass over the alfalfa or the medium brome grass.

It is noted that there is an increase of TDN, DE, and digestible dry matter from alfalfa hay to the low brome grass hay, and from the low to the medium brome grass hay. These differences are statistically significant between most of the hays. The medium and high brome grass hays exhibit approximately the same values in the three methods of nutritive evaluation. The nitrogen balance during all individual periods was positive, the amount of nitrogen stored per period per lamb being closely related to the content of digestible protein of the hay. The differences between the average amounts of nitrogen stored per period were not significant.

Reed canary grass trial ('57). Since the reed canary grass hays used in this trial were grown during the spring and summer of 1957, which was a very dry season, pure stands were very hard to maintain. The second growth on the plots without nitrogen fertilization was insufficient to be harvested, consequently first cutting hay was substituted. In addition, the hay fertilized with 100 pounds of nitrogen per acre was damaged by rain. Although these variables were expected to greatly disguise the effect of nitrogen fertilization upon the nutrient constituents of the hays and their digestibilities, the comparison of TDN and DE determined from these hays is not affected.

As in the previous trial, increasing nitrogen fertilization of the reed canary grass increased the content of protein, ether extract, and moisture, while the nitrogen-free extract content was decreased. No consistent relationship with increased nitrogen fertilization was observed in the content of fiber, ash, and gross energy. The average digestion coefficients of the nutrient components in the reed canary grass hays are presented in table 1. It may be observed that higher nitrogen fertilization again increased the protein digestion coefficients, and that the fiber digestibility of the alfalfa was again considerably below that of all the grass hays. The medium reed canary grass hay exhibits the lowest apparent digestibilities of fiber, ether extract, nitrogen-free extract, and energy of the reed canary grass hays, and also the lowest values of TDN, DE, and digestible dry matter. The results of the digestibilities for fiber, nitrogen-free extract, energy, TDN and dry matter within the limits of this experiment indicate that the first cutting of reed canary grass is superior to either the medium or high level nitrogen application groups. The digestion coefficients for the latter groups may have been effected by either the contamination of orchard grass or the climatic conditions at the time of harvest or both. Nitrogen balances were positive for all the individual periods, the amounts of nitrogen storage being dependent on digestible protein content of the hays and the amounts ingested.

As expected, a close relationship existed between the averages of TDN and DE. A much closer relationship was observed between the pairs of values for each individual digestion trial. Using the latter data, correlation coefficients of $+ 0.95$ and $+ 0.87$ were calculated for the brome grass and the reed canary grass trials, respectively. Both correlation coefficients were significant at the 1% level of probability. Using the least squares method, the regression equation of $Y = 189.82 + 42.87\,X$ was calculated for the brome grass trial, and $Y = 758.42 + 32.98\,X$ for the reed canary grass trial, where Y is the DE value expressed in Calories per gram of feed and X is the TDN percentage. Within the limits of this investiga-

tion, the values calculated from these two equations are quite similar.

SUMMARY

Brome grass hays grown with 25, 125, and 225 pounds of nitrogen per acre, and reed canary grass hays grown with 0, 100, and 200 pounds of nitrogen per acre, were compared to alfalfa hay in two total collection digestion trials using 4 lambs each, to determine the effect of nitrogen fertilization on the digestibility of the hays. Both total digestible nutrients (TDN) and digestible energy (DE) were determined and compared as to their quantitative relationship.

Increasing nitrogen fertilization caused an increase in the protein content from 7.9 to 15.2% in the brome grass hays and from 12.6 to 20.1% in the reed canary grass hays. At the higher levels of nitrogen fertilization on brome grass there was also an increase in the gross energy and ether extract content while the fiber and nitrogen-free extract decreased. No similar trend of the crude components was observed with the reed canary grasses, which may be due to the several variables encountered in the production of these hays.

A statistically significant increase of the apparent digestibility of protein with increasing nitrogen fertilization was observed with both grass species. Energy digestion coefficients are also increased at the higher levels of fertilization in the case of brome grass. These differences were less marked and not significant between all levels. The fiber digestion coefficients of all grass hays were considerably higher than those of alfalfa hay.

The correlation coefficients between TDN and DE were + 0.95 for the brome grass trial and + 0.87 for the reed canary grass trial and significant at the 1% level of probability. This indicated the close relationship of the two methods of feed evaluation. Regression equations between TDN and DE were $Y = 189.82 + 42.87\,X$ for the brome grass trial, and $Y = 758.42 + 32.98\,X$ for the reed canary grass trial, where Y is DE in Calories per gram and X is the TDN percentage of the hay.

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THE GASTROINTESTINAL DIGESTION OF FAT IN DOGS FED TRIGLYCERIDES, PARTIAL GLYCERIDES AND FREE FATTY ACIDS

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A number of workers have studied pancreatic lipase hydrolysis of triglyceride fat *in vitro* in recent years (Frazer and Sammons, '45; Desnuelle, '51; Borgstrom, '54). In general, they found that considerable quantities of monoglycerides, diglycerides, triglycerides and free fatty acids were present in the test tube after relatively long periods of time. It has also been demonstrated that appreciable amounts of these glycerides and free fatty acids are present in lipid recovered from the intestinal tract following the feeding of triglyceride fat (Mattson et al., '52; Desnuelle and Constantin, '52). It appears that lipolysis *in vivo* and *in vitro* proceeds in a stepwise fashion from triglyceride to diglyceride to monoglyceride to glycerol with the liberation of fatty acid at each step. However, it has been shown that hydrolysis of triglycerides *in vitro* proceeds so slowly that a complete hydrolysis to free fatty acids and glycerol does not occur in the time it normally takes fat to traverse the small intestine. These results suggest that both glycerides and free fatty acids may be absorbed by the small intestine. This is contrary to the concept of fat digestion based on a complete breakdown of triglycerides to free fatty acids and their absorption as such (Verzar and McDougall, '36). In the present paper an attempt is made to determine whether a parallelism does exist between lipolysis *in vivo* and *in vitro*.

METHODS

The experimental procedure has been described (Knoebel and Nasset, '57). Dogs fasted at least 24 hours were fed a test meal, killed after three hours and contents removed from the stomach, duodenum, jejunum and ileum. The duodenum represented about 8% and the jejunum and ileum each approximately 46% of the length of the small intestine.

Free fatty acids were titrated in 95% ethanol with alcoholic 0.1N KOH to the phenolphthalein endpoint. Monoglyceride content was determined by the iodometric technique of Pohle and Mehlenbacher ('50). For reasons given elsewhere (Knoebel and Nasset, '57) both 1- and 2-monoglycerides are considered to be included in the final values presented in this work. The part of the recovered lipid not accounted for as free fatty acids or monoglycerides was assumed to consist of diglycerides and triglycerides. That this assumption is valid for the most part is supported by the finding of Desnuelle and Constantin ('52) that the amounts of phospholipid and cholesterol, free and esterified, present in the lipid recovered from the intestine of rats fed olive oil is very small compared to the quantities of glycerides and free fatty acids present.

Knoebel and Nasset ('57) reported that the composition of lipid recovered from various segments of the small intestine of dogs fed cottonseed oil triglycerides closely resembled that produced by the action *in vitro* of pancreatic lipase on triolein in the presence of calcium ions and bile salts (Desnuelle, '51). This suggested that triglycerides may be hydrolyzed in much the same manner *in vivo* as *in vitro*. In order to investigate further whether such a relationship exists, 4 lipid mixtures were fed to dogs. Each lipid mixture had a composition (table 1) similar to that which presumably exists at a certain time during the first hour of hydrolysis of triolein *in vitro* as reported by Desnuelle ('51). A test meal contained 25 gm of a lipid mixture, 25 gm of sucrose, 25 gm of casein and 5 gm of cellulose flour. Two dogs were fed a test meal

consisting of 40 gm of cottonseed oil triglycerides and 100 gm of sucrose. Data obtained from these two dogs were combined with previously published data (Knoebel and Nasset, '57) obtained from 5 dogs fed the same test meal. These combined data are included in the present work in order to give a more complete picture of the manner in which lipid is digested.

The diglycerides and monoglycerides [1] used in these studies were commercially prepared from cottonseed oil triglycerides and contained the same fatty acids in the same proportions as found in cottonseed oil triglycerides. The free fatty acids were prepared by hydrolysis of cottonseed oil triglycerides. All of the lipids used were 95% pure or better.

RESULTS AND DISCUSSION

The weights of lipid recovered from the stomach and various portions of the small intestine of dogs fed the different test meals are given in table 2. Since the test meals containing the 4 lipid mixtures were identical in every respect except for lipid composition, comparisons of the gastric evacuation of lipid could be made. As calculated from the amount of lipid fed and the amount of lipid recovered from the stomach at the end of three hours, gastric evacuation of lipid in dogs fed the lipid mixtures containing 14.4, 20.9, 30.5 and 46.2% of free fatty acids was 49, 49, 46 and 49%, respectively. Thus, variations in gastric emptying time probably did not affect intestinal digestion and absorption of lipid. Since the composition of the test meal containing cottonseed oil triglycerides was considerably different from that of the test meals containing the lipid mixtures, a fair comparison of the gastric evacuation of cottonseed oil triglycerides (38%) can not be made with that of the lipid mixtures. In most cases there were no significant differences in the weights of lipid recovered from corresponding segments of intestine of dogs

[1] The monoglycerides and diglycerides were provided by the Distillation Products Industries, Incorporated, Rochester, New York.

TABLE 1

Composition [1] *of dietary lipid*

TEST MEAL		FREE FATTY ACIDS	MONO-GLYCERIDES	DI-GLYCERIDES	TRI-GLYCERIDES	TIME OF HYDROLYSIS *In Vitro* [2]
		%	%	%	%	*min.*
Cottonseed oil	(7)[3]	0.1	0.4	—	99.5	0
14.4% FFA [4]	(8)	14.4	4.4	7.3	73.9	10
20.9% FFA	(7)	20.9	7.5	15.5	56.1	15
30.5% FFA	(7)	30.5	12.1	18.4	39.0	25
46.2% FFA	(7)	46.2	15.5	28.5	9.8	51

[1] The composition of the lipid mixtures was similar to that produced at different times during the first hour of the hydrolysis of triolein *in vitro* as reported by Desnuelle ('51). All values represent percentage composition by weight.
[2] Time at which the lipid mixtures were present during the hydrolysis of triolein *in vitro*.
[3] Number of dogs fed the test meal given within parentheses.
[4] Test meals are named for free fatty acid (FFA) content.

TABLE 2

Weights of total lipid recovered from gastrointestinal contents of the dog

TEST MEAL	FED		STOMACH	DUODENUM	JEJUNUM	ILEUM	TOTAL SMALL INTESTINE
	gm		*gm*	*mg*	*mg*	*mg*	*mg*
Cottonseed oil	40	(7)[1]	24.7 ± 2.2 [2]	284 ± 129	712 ± 217	257 ± 77	1244 ± 372
14.4% FFA [3]	25	(8)	12.7 ± 0.8	416 ± 125	646 ± 154	204 ± 53	1287 ± 209
20.9% FFA	25	(7)	12.8 ± 0.8	201 ± 44	549 ± 77	155 ± 50	991 ± 129
30.5% FFA	25	(7)	13.4 ± 0.9	307 ± 63	789 ± 109	264 ± 95	1312 ± 257
46.2% FFA	25	(7)	12.8 ± 0.4	428 ± 100	957 ± 169	329 ± 113	1714 ± 273

[1] Number of samples given within parentheses.
[2] Mean ± standard error of the mean.
[3] Test meals are named for free fatty acid (FFA) content.

fed the various test meals. In a few instances lipid sufficient for the analyses could not be obtained from the duodenum and the ileum.

The composition of lipid recovered from the stomach and intestinal segments after feeding the various test meals is given in table 3. It was necessary to determine the extent to which fat derived from a particular test meal was digested in the stomach in order that intestinal lipolysis could be more fully evaluated for the same test meal. Gastric lipolysis is not extensive enough to complicate the interpretation of results obtained on intestinal lipolysis.

As lipid passed through the small intestine hydrolysis progressed steadily, as manifested by the increased percentages of free fatty acids and monoglycerides. On the basis of computation by difference a corresponding decrease occurred in the percentage of combined diglycerides and triglycerides. A decrease in the percentages of free fatty acids and monoglycerides and an increase in the percentage of combined diglycerides and triglycerides occurred in the duodenum of dogs fed the lipid mixture containing 46.2% of free fatty acids. It would appear that a synthesis of higher glycerides through esterification of free fatty acids with partial glycerides took place in the duodenum of these dogs. Pancreatic lipase may catalyze synthetic reactions as shown by Borgstrom ('52) who reported that a synthesis of new glyceride ester bonds with free fatty acids occurred simultaneously with hydrolysis in the small intestine of the rat.

The composition of lipid recovered from the duodenum of dogs fed the lipid mixture containing 30.5% of free fatty acids was not significantly different from that of the gastric lipid. Hydrolysis in the duodenum of dogs fed cottonseed oil triglycerides and the lipid mixtures containing 14.4 and 20.9% of free fatty acids decreased as the proportion of free fatty acids to glycerides increased in these test meals. Thus, if the composition of the intestinal lipid reflects the manner in which lipase acts, these results suggest that as the proportion of free fatty acids to glycerides increased in the dietary

TABLE 3

Composition [1] *of lipid recovered from the gastrointestinal contents of the dog compared with the composition of lipid produced by pancreatic lipase hydrolysis of triolein in vitro*

SAMPLE FROM		FREE FATTY ACIDS	MONOGLYCERIDES		DIGLYCERIDES AND TRIGLYCERIDES	
		in vivo	*in vitro* [2]	*in vivo*	*in vitro* [2]	*in vivo* [3]
		%	%	%	%	%
Test meal		0.1		0.4		99.5 [4]
Stomach	(7) [5]	2.9 ± 0.8 [6]	1.0	1.0 ± 0.2	96.1	96.1 ± 1.0
Duodenum	(6)	19.4 ± 3.5	7.0	5.2 ± 0.5	73.6	75.4 ± 5.0
Jejunum	(7)	32.3 ± 4.4	11.9	8.9 ± 1.1	55.8	58.8 ± 5.2
Ileum	(7)	43.7 ± 5.3	14.8	13.2 ± 1.4	41.5	43.1 ± 6.0
Test meal		14.4		4.4		81.2
Stomach	(8)	16.8 ± 0.3	6.1	4.4 ± 0.1	77.1	78.8 ± 0.3
Duodenum	(8)	25.3 ± 2.3	9.3	11.6 ± 1.7	65.4	62.7 ± 3.8
Jejunum	(8)	35.8 ± 1.4	13.3	14.8 ± 1.1	50.9	49.4 ± 2.0
Ileum	(6)	38.3 ± 4.6	13.8	18.7 ± 1.1	47.9	43.0 ± 3.8
Test meal		20.9		7.5		71.6
Stomach	(7)	22.9 ± 0.5	8.2	7.6 ± 0.1	68.9	69.5 ± 0.5
Duodenum	(6)	25.3 ± 1.5	9.2	8.0 ± 0.8	65.5	66.7 ± 1.9
Jejunum	(7)	37.2 ± 2.9	13.6	14.9 ± 0.7	49.2	47.8 ± 3.4
Ileum	(6)	42.2 ± 3.6	14.4	15.8 ± 1.3	43.4	42.0 ± 4.5
Test meal		30.5		12.1		57.4
Stomach	(7)	31.8 ± 0.2	11.8	11.8 ± 0.2	56.4	56.4 ± 2.3
Duodenum	(6)	30.2 ± 2.1	11.2	10.9 ± 1.0	58.6	58.9 ± 2.3
Jejunum	(7)	38.1 ± 2.3	13.7	12.7 ± 0.6	48.2	49.2 ± 1.8
Ileum	(6)	45.9 ± 4.7	15.3	16.0 ± 1.6	38.9	38.1 ± 4.8
Test meal		46.2		15.5		38.3
Stomach	(7)	45.6 ± 0.2	15.1	14.8 ± 0.4	39.3	39.6 ± 0.5
Duodenum	(7)	34.8 ± 3.0	12.9	10.0 ± 2.7	52.3	55.2 ± 3.8
Jejunum	(7)	37.9 ± 1.5	13.7	14.1 ± 0.4	48.4	48.0 ± 1.4
Ileum	(6)	45.4 ± 9.0	15.0	14.5 ± 1.7	39.6	40.1 ± 7.8

[1] All values represent percentage composition by weight.

[2] The percentages of monoglycerides and combined diglycerides and triglycerides *in vitro* were obtained from the data of Desnuelle ('51). The percentages of free fatty acids in lipid recovered from the stomach and intestinal segments of dogs fed the various test meals were used as a reference for obtaining these values.

[3] Combined diglycerides and triglycerides were determined by difference between other fractions and 100%.

[4] Cottonseed oil triglycerides. Other values for combined diglycerides and triglycerides present in the test meal lipid represent mixtures of these two lipid components in the proportions in which they exist at certain times during the hydrolysis of triolein *in vitro* as reported by Desnuelle ('51).

[5] Number of samples given within parentheses.

[6] Mean ± standard error of the mean.

lipids, a gradual change from hydrolysis to synthesis was effected in the duodenum by the action of pancreatic lipase. However, it should be emphasized that the composition of intestinal lipid depends not only on the action of lipase, but on the absorptive mechanism as well. Depending on the form in which fat is actually absorbed, a variety of alternative interpretations could be offered in explanation of the alterations in lipid composition observed in these experiments.

Knoebel and Nasset ('57) reported that the lipid components were present in very similar proportions in lipid recovered from the ileum of dogs fed test meals containing either cottonseed oil triglycerides or the 1-monoester of linoleic acid. They suggested that a certain lipid system tends to be formed in the intestine by the action of lipolytic enzymes and that the ease with which such a system is formed depends on the type of lipid fed. The results in table 3 show that there was a definite tendency for a common lipid system to be formed in the proximal portion of the small intestine of dogs fed a variety of test meal lipids. Although the lipids recovered from the duodenum of dogs fed the various test meals were different in composition, they were much more alike than the corresponding test meals. Depending on the composition of lipid fed, this trend toward a similarity of composition in the duodenum appeared to be effected primarily by hydrolysis in some instances and synthesis in others. Hydrolytic reactions predominated in the jejunum and ileum. The composition of lipid recovered from these segments of intestine was very similar regardless of the lipid fed. These results suggest that once a common lipid system was formed in the duodenum or proximal jejunum, hydrolysis proceeded at about the same rate throughout the remainder of the small intestine of dogs fed the various test meals.

The results obtained by the analyses of the contents of the duodenum, jejunum and ileum represent increasing time intervals with respect to the extent to which lipid was acted on by lipase. Thus, comparisons can be made between the composition of lipid recovered in these experiments *in vivo*

and that obtained at different times during the hydrolysis of triolein *in vitro* as reported by Desnuelle ('51). In order to make such comparisons, the percentages of free fatty acids in lipid recovered from the stomach and various parts of the small intestine were used as a base for relating the composition of lipid *in vivo* and *in vitro*. For example, the lipid recovered from the duodenum of dogs fed cottonseed oil triglycerides contained 19.4% of free fatty acids. By referring to a graph constructed from the data of Desnuelle ('51) it was possible to determine the percentages of monoglycerides and combined diglycerides and triglycerides *in vitro* that existed at the time the lipid mixture in the test tube also contained 19.4% of free fatty acids. These comparisons were made for all portions of the gastrointestinal tract studied and are given in table 3.

Exact comparisons can be made between studies *in vivo* and *in vitro* after feeding cottonseed oil triglycerides, since in both instances triglycerides were subjected to the action of pancreatic lipase. In order to make accurate comparisons between the two types of studies after feeding the various lipid mixtures, the composition of lipid recovered from the intestinal segments should be compared with that produced by the action of pancreatic lipase on these same lipid mixtures *in vitro*. Since these latter data were unavailable, such comparisons could not be made. However, if the action of pancreatic lipase *in vivo* and *in vitro* is the same, it was felt that some understanding of the digestive processes would be gained by using the method of comparison described above.

The percentages of lipid components recovered from the various intestinal segments of dogs fed cottonseed oil triglycerides and the lipid mixtures containing 14.4 and 20.9% of free fatty acids were similar to those obtained during the first hour of hydrolysis of triolein *in vitro*. The only exception was for the ileum of the dogs fed the lipid mixture containing 14.4% of free fatty acids. The method used for making comparisons of lipid composition between the studies *in vivo* and *in vitro* for the duodenum of dogs fed the lipid

mixtures containing 30.5 and 46.2% of free fatty acids necessitated referring to the data of Desnuelle in the direction representing synthesis. The composition of lipid recovered from this segment of intestine, where synthesis appeared to predominate, was similar to that obtained during the hydrolysis of triolein *in vitro*. The proportion of lipid components in the jejunum and ileum, where the action of pancreatic lipase favored hydrolysis, continued to resemble closely that obtained during the study *in vitro*. Thus, the composition of lipid recovered from these segments of intestine compared favorably with the data of Desnuelle, first in a synthetic, and then in a hydrolytic direction. It was also interesting to note that the composition of lipid recovered from the ileum of dogs fed the lipid mixture containing 46.2% of free fatty acids was practically the same as that of the gastric lipid. These results suggest that the catalytic properties of pancreatic lipase are such that the synthetic reactions may be considered, for the most part, a reversal of the hydrolytic reactions.

Since the composition of lipid recovered from the intestine after feeding cottonseed oil triglycerides was similar to that obtained by the hydrolysis of triolein *in vitro,* it would appear that the manner in which triglycerides are hydrolyzed is the same in both types of studies. Further support for this idea is provided by the fact that the same was true for dogs fed lipid mixtures, the composition of which represented 4 different stages in the digestion of triglycerides *in vitro*. If the comparisons between the action of pancreatic lipase *in vivo* and *in vitro* are valid, it might be argued that all lipid components are absorbed in the proportions in which they exist as the result of digestion at any point in the intestine. A non-specific absorption such as this would be one way by which the composition of lipid in the intestine could be maintained similar to that in the test tube, which lacks an absorptive mechanism. If, as suggested by Verzar and McDougall ('36), only free fatty acids are absorbed in the

intestine, the resulting similarity in the composition of lipid between the studies *in vivo* and *in vitro* is coincidental.

SUMMARY

Dogs were fed cottonseed oil triglycerides and 4 lipid mixtures, the composition of which represented various stages in the hydrolysis of triolein *in vitro*. The composition of lipid recovered from the various parts of the small intestine of dogs fed these test meals closely resembled that obtained during the hydrolysis of triolein *in vitro*. Triglycerides may be hydrolyzed, therefore, in much the same manner *in vivo* as *in vitro*.

Although the composition of the dietary lipids was quite different, there was a tendency for a common lipid system to be formed in the proximal portion of the small intestine (free fatty acids, 27%; monoglycerides, 9%; di- and triglycerides, 64%) of dogs fed these test meals. Hydrolytic reactions predominated in the duodenum of dogs fed lipids which contained a high proportion of glycerides to free fatty acids. The percentages of free fatty acids and monoglycerides decreased and the percentage of combined diglycerides and triglycerides increased in lipid recovered from the duodenum of dogs fed lipid containing a high proportion of free fatty acids to glycerides. This suggested that a synthesis of higher glycerides occurred in the duodenum of these dogs. Hydrolytic reactions predominated in the jejunum and ileum of dogs fed the various test meals. The composition of lipids recovered from the jejunum (free fatty acids, 36%; monoglycerides, 13%; di- and triglycerides, 51%) was very similar regardless of the lipid fed. The same was true for lipid recovered from the ileum (free fatty acids, 43%; monoglycerides, 16%; di- and triglycerides, 41%).

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AMINO ACID BALANCE AND IMBALANCE[1]

I. DIETARY LEVEL OF PROTEIN AND AMINO ACID IMBALANCE

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The concept of amino acid balance is founded on a knowledge of the relationship between the amino acid composition of a protein and its biological value. A protein that provides amino acids in roughly the proportions in which they are required by the body is termed a balanced protein and has a high biological value: a protein that is low in one or more of the indispensable amino acids is termed an *unbalanced* protein and has a lower biological value. The more unbalanced a protein is, the lower the efficiency with which it is used and the greater the amount needed in a diet to satisfy the amino acid requirements (Block and Mitchell, '46-'47; Oser, '51; Almquist, '53; Mitchell, '54; Flodin, '53, '57).

The term amino acid *imbalance* has arisen from studies in which adverse effects, beyond the expected fall in the efficiency of protein utilization, have been observed when the protein of a diet, usually one low in protein, has been thrown out of balance by the addition of amino acids or a quantity of an unbalanced protein. In order to reverse these adverse effects, such as retarded growth or an accumulation of liver fat, a supplement of the amino acid that is most limiting in the diet must be provided. Thus, an amino acid imbalance,

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besides causing a fall in the efficiency of nitrogen utilization, also causes an increase, specifically, in the need for the most limiting amino acid, (Harper, '58; Salmon, '58).

Although Salmon ('54) has studied the effect of dietary additions of gelatin on the tryptophan requirement there have been relatively few studies of a quantitative nature on the effects of amino acid imbalances. In fact, much of our information about such imbalances has been obtained from studies designed for other purposes.

There is even some difference of opinion about the use of the term amino acid imbalance (Harper, '58; Salmon, '58). For the present, an amino acid imbalance will be defined as any change in the proportions of the amino acids in a diet that result in *an adverse effect which can be prevented by supplementing the diet with a relatively small amount of the most limiting amino acid or acids.* This leaves open the question of whether there are different types of amino acid imbalances, i.e., whether imbalances caused by adding relatively small amounts of one or two amino acids to a diet (Hankes et al., '49; Deshpande et al., '58a) are identical with those produced by adding a relatively large quantity of a protein or of an amino acid mixture lacking a single amino acid (Salmon, '54; Deshpande et al., '55, '58 a, b; Sauberlich, '56; Harper, '59). It excludes, however, those conditions described as antagonisms and toxicities (Harper, '58), in which adverse effects are caused by the addition of a fairly large excess of a single amino acid, and which are not known to be prevented by a relatively small supplement of the amino acid that is most limiting for growth.

The objectives of the investigation to be reported in this and in subsequent papers in this series are: one, to obtain both qualitative and quantitative information about the effects of changes in amino acid balance in an effort to provide a link between the observations on the relationship between the amino acid composition of a protein and its biological value and the observations on alterations in the proportions of amino acids in a diet that lead to amino acid imbalances;

and, two, to extend the previous observations on the physiological and metabolic effects of amino acid imbalances (Sauberlich and Salmon, '55; Deshpande et al., '58b; Kumta et al., '58).

The original purpose of the experiments reported in this paper was to study quantitatively the effect of the dietary level of protein on the severity of an amino acid imbalance. As a result of the initial experiments an hypothesis was developed which appeared to explain why dietary additions that caused quite severe imbalances in low protein diets were almost without effect when the protein level was sufficiently high to satisfy the amino acid requirements of the experimental subjects. The hypothesis was based on the fact that the growth response to a given increment of the amino acid that is most limiting in a diet diminishes as the growth rate approaches a maximum (fig. 2). From this it followed that if the requirement for the limiting amino acid were increased by a constant amount when a quantity of an amino acid mixture causing an imbalance was added to a diet, then the growth-retarding effect of such an addition should diminish as the dietary level of protein approached adequacy. The hypothesis could be tested experimentally by determining the growth rates of groups of animals ingesting diets containing: (1) a constant level of balanced protein but increasing increments of an unbalanced protein; (2) a constant level of an unbalanced protein or amino acid mixture but increasing increments of balanced protein; (3) either of the above with increments of the amino acid most limiting for growth. The results of such experiments appeared to support the hypothesis.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, 21 days old and weighing from 40 to 50 gm were fed the basal diet for three days. They were then separated into groups of 5 rats each and were maintained in individual suspended cages with ½ in. mesh screen bottoms. The average initial

weights for the groups within each experiment did not differ by more than 1 gm. The rats were fed ad libitum and were weighed at least twice weekly during the two-week experimental periods.

The percentage composition of the basal diet was as follows: casein, 6.0; gelatinized corn starch, 83.6; corn oil, 4.5; mineral mixture, 5.0; choline chloride 0.15; fat-soluble vitamin mixture in corn oil, 0.5; and water soluble vitamin mixture in sucrose, 0.25. The mineral mixture was devised in collaboration with Dr. R. E. Boldt on the basis of a review of the rat requirements by Cuthbertson ('57). It had the following percentage composition: $CaCO_3$, 29.29; $Ca\ HPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.623; $CuSO_4 \cdot 5H_2O$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.02; KI, 0.0005; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025. The water-soluble vitamin mixture provided in milligrams per 100 gm of diet: thiamine·HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; calcium pantothenate, 2.0; pyridoxine ·HCl, 0.25; vitamin K (menadione), 0.05; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.002; inositol, 10.0; and ascorbic acid, 5.0. The fat-soluble vitamin mixture provided per 100 gm of diet: α-tocopherol, 10.0 mg; vitamin A, 400 I.U.; and vitamin D, 200 I.U. The ascorbic acid was included in the vitamin mixture to minimize the destruction of thiamine (Kandutsch and Baumann, '53) and all rations were refrigerated. Changes in the protein level and all additions of amino acids, as indicated in the tables of results, were compensated for by adjusting the percentage of carbohydrate.

RESULTS

In order to determine the extent to which the severity of an amino acid imbalance was influenced by the level of the protein causing the imbalance, various levels of gelatin were added to diets containing either 6 of 8% of casein supplemented with 0.3% of DL-methionine. This procedure is known to produce an imbalance involving tryptophan even if the

supply of niacin is adequate (Salmon, '54). As is shown in table 1, the growth of rats fed a diet containing 6% of casein supplemented with methionine was stimulated by the addition of threonine. Gelatin, which contains threonine but not tryptophan, stimulated growth when added at a level of only 3%; however, when increments greater than 3% of gelatin were added, the rate of gain fell off until, with the addition of 12% of gelatin the rate of gain was only 13 gm in two weeks, considerably less than that of the group fed only the

TABLE 1

Effect of gelatin level on rate of gain of rats fed on diets containing 6 or 8% of casein supplemented with 0.3% of DL-*methionine*

Gelatin	SUPPLEMENTS		WEIGHT GAIN	
	DL-threonine	DL-tryptophan	6% casein	8% casein
%	%	%	*gm/2 wks.*	*gm/2 wks.*
—	—	—	22 ± 3 [1]	53 ± 4 [1]
—	0.2	—	43 ± 3	—
3	—	—	31 ± 4	—
6	—	—	23 ± 3	52 ± 3
9	—	—	20 ± 2	40 ± 5
12	—	—	13 ± 2	34 ± 4
12	—	0.2	51 ± 4	—
15	—	—	—	24 ± 3
15	—	0.2	—	77 ± 5

[1] Standard error of the mean.

basal diet. The addition of tryptophan, the most limiting amino acid, not only prevented the growth retardation but stimulated growth above that obtained with the basal diet. A similar trend was seen when the diet contained 8% of casein, but the values in each case were higher.

The influence of the level of dietary protein on the imbalance caused by adding an amino acid mixture lacking threonine to diets containing casein supplemented with methionine is shown in figure 1. The amino acid mixture contained 3.45% of amino acids, equivalent to the amounts of the L-isomers of these indispensable amino acids in 6% of casein. This mixture, which induces an amino acid imbalance that

is readily corrected by a supplement of threonine (Harper, '59), caused the greatest depression in growth rate when the diet contained 6% of casein. The depression was less when the casein content of the diet was dropped to 4% and also when it was increased above 6%. Very little growth depression was observed when the diet contained 15% of casein.

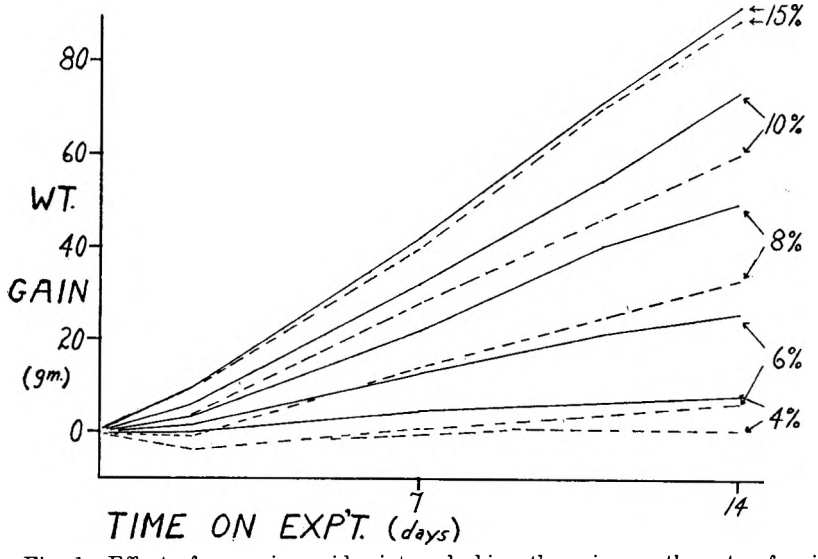

Fig. 1 Effect of an amino acid mixture lacking threonine on the rate of gain of rats fed on diets containing different levels of casein supplemented with 0.3% of DL-methionine. —— no addition; - - - - amino acid mixture lacking threonine. The amino acid mixture provided in per cent of the diet: DL-tryptophan, 0.15; L-leucine, 0.54; DL-isoleucine, 0.8; DL-valine, 0.82; L-histidine·HCl, 0.22; DL-phenylalanine, 0.34; L-lysine·HCl, 0.58.

The next experiment provided information about the effect of 4% of the amino acid mixture lacking threonine on the need for threonine by rats fed a diet containing 6% of casein supplemented with 0.3% of DL-methionine. The results are presented in table 2. The amino acid mixture lacking threonine depressed the growth rate, as before, and somewhere between 0.025 and 0.05% of L-threonine had to be

added to overcome the growth depression. These results suggest that this percentage of the threonine in the original diet was made unavailable to the animal because of the imbalance. Increments of threonine greater than this stimulated growth above that of the control group indicating that threonine was by far the most limiting amino acid in this diet.

TABLE 2

Effect of 4% of an amino acid mixture lacking threonine on the need for threonine by rats fed a diet containing 6% of casein supplemented with 0.3% of DL-*methionine*

Casein	DIET		WEIGHT GAIN
	A. A. mix [1]	L-threonine	
%	%	%	*gm/2 wks.*
6	—	—	18 ± 3 [2]
6	4	—	10 ± 1
6	4	0.025 [2]	11 ± 2
6	4	0.05	21 ± 3
6	4	0.075	27 ± 4
6	4	0.1	42 ± 2
6	4	0.15	51 ± 4

[1] For the composition of the amino acid mixture see figure 1. The quantity of each of the amino acids was increased proportionally.

[2] Standard error of the mean.

The results presented in figure 1 indicate that the magnitude of the growth depression caused by the addition of an unbalanced amino acid mixture depends upon the level of protein in the diet. This suggests that the magnitude of the effect can be related to the standard growth response curve obtained when gain in weight is plotted against the response to increasing increments of protein or of the limiting amino acid in the diet. Also, from the results in table 2, it is evident that close to an additional 0.05% of L-threonine was required in the diet to overcome the growth retardation caused by the amino acid mixture lacking threonine or, as was concluded from these results, this percentage of the threonine in the original diet was made unavailable to the animal when the amino acid imbalance was created. If an amino acid

mixture causing an imbalance increases the need of the animal for the limiting amino acid by a constant percentage regardless of the original level of protein in the diet, then, although the magnitude of the growth depression caused by adding a stated amount of an unbalanced amino acid mixture to a diet would depend upon the adequacy of the diet, the growth depression should be prevented by the same level of the limiting amino acid in each case.

This is illustrated by figure 2 which has been constructed from data obtained by Armstrong ('54) in an investigation of the phenylalanine requirement of the rat. It also indicates the basis for the final experiments in this study. The cross-hatched areas indicate that an amino acid mixture which increased the requirement for the limiting amino acid by 0.1% would cause a much greater growth depression when added to a diet supporting a rate of gain that fell on the steeper part of the curve than it would if it were added to a diet that was nearly adequate (upper part of the growth response curve) or was severely inadequate (lower part of the growth response curve).

The final experiments were conducted to determine how closely experimental results would fit this hypothesis. In order to increase the severity of the imbalance, and on the basis of the results obtained in experiment 1, the amount of the amino acid mixture lacking threonine that was added to the diet was increased to 6%; however, the relative proportion of each of the amino acids in the mixture remained unaltered (see fig. 1). The results of the experiment are presented in table 3. Three levels of casein, supplemented with 0.3% of DL-methionine in each case, were used in this experiment and for the sake of economy DL-threonine (a 50–50 mixture of D-allo-threonine and L-threonine) was substituted for L-threonine. With each level of casein, 4, 7 and 10%, the amino acid mixture lacking threonine caused a growth depression. The magnitude of the growth depression was greatest when the diet contained 7% of casein and was less if the diet contained either 4 or 10% of casein. Regardless

of the level of casein the growth depression was not prevented by the addition of 0.05% of DL-threonine but was completely prevented by an addition of 0.1%. These results are what would have been expected on a theoretical basis.

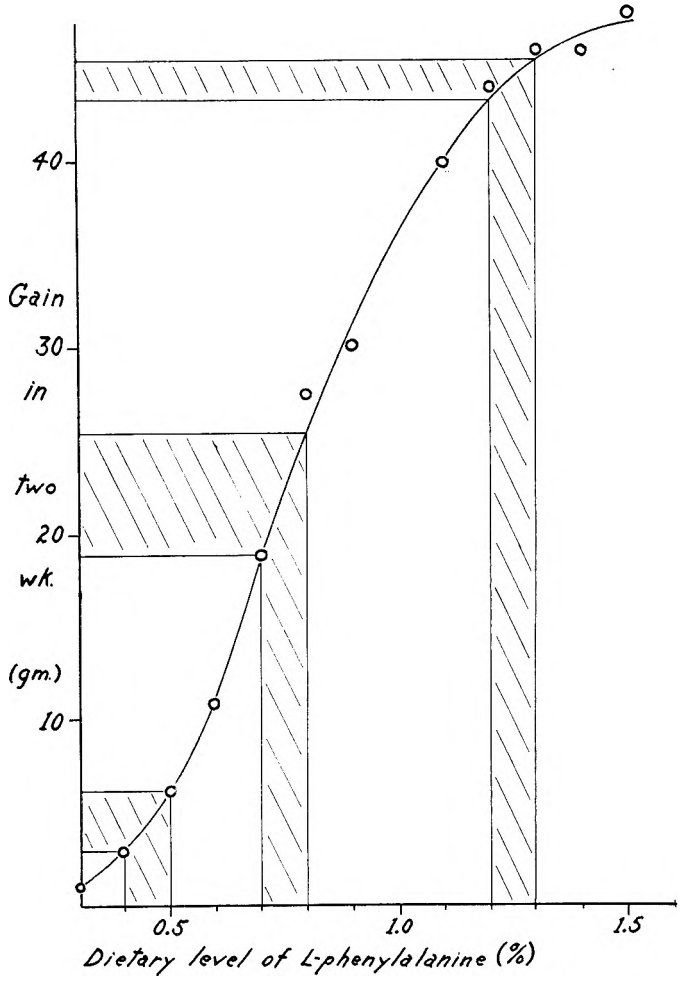

Fig. 2 Growth response curve. Gain in weight plotted against phenylalanine content of diet; taken from data of Armstrong ('54). Cross-hatched areas indicate the effect on the rate of gain of removing 0.1% of L-phenylalanine from the diet.

Although the magnitude of the growth depression was greatest when the diet contained 7% of casein, i.e., when the diet supported about half the maximum rate of gain, it is of interest to look at the figures expressed on a percentage basis.

TABLE 3

Effect of 6% of an amino acid mixture lacking threonine on the need for threonine by rats fed on diets containing various levels of casein supplemented with 0.3% of DL-*methionine*

Casein	DIET		WEIGHT GAIN	% OF CONTROL GROWTH
	A. A. mix [1]	DL-threonine		
%	%	%	*gm/2 wks.*	
4	—	—	9 ± 1 [2]	
4	6	—	1 ± 2	11
4	6	0.05	5 ± 1	56
4	6	0.1	9 ± 1	100
7	—	—	41 ± 2	
7	6	—	19 ± 3	46
7	6	0.05	27 ± 2	66
7	6	0.1	41 ± 4	100
10	—	—	73 ± 4	
10	6	—	61 ± 3	83
10	6	0.05	65 ± 4	89
10	6	0.1	76 ± 5	104

[1] For the composition of the amino acid mixture see figure 1. The quantity of each of the amino acids was increased proportionally.

[2] Standard error of the mean.

When the diet contained 4% of casein, the amino acid mixture lacking threonine depressed the growth rate to 11% of that of the control group; with 7% of casein in the diet, the value was 46%; and with 10% of casein, 83%. Thus, the effect of the imbalance was most severe when the diet contained only 4% of protein.

DISCUSSION

The procedure used in these experiments to induce an amino acid imbalance consisted of adding a fairly large quantity of an amino acid mixture or of a protein lacking a single amino acid to a diet that contained an inadequate amount of protein. This has proven to be an effective and

consistent method of creating amino acid imbalances (Salmon, '54; Deshpande et al., '55, '58a,b; Sauberlich, '55; Harper, '59). There are, however, some cases in which a relatively small addition of amino acids, usually the amino acid that is second most limiting in the diet, causes an unexpectedly severe imbalance (Hankes et al., '49; Henderson et al., '53; Deshpande et al., '58a). The question therefore arises as to whether both of these situations are examples of a general phenomenon or whether they represent two different conditions. Although some preliminary observations suggest that there are differences between them[2] a definitive answer must await the completion of further experiments.

These observations also pose a question concerning the method of determining amino acid requirements. The usual procedure is to provide all of the amino acids except one in excess and then to measure the response to increasing increments of that one. This is very similar to the procedure that was used to create amino acid imbalances in this study. It would seem advisable for the amino acid balance to be maintained as closely as possible to the ideal if minimum values for amino acid requirements are to be obtained.

Also, in the studies in which amino acid requirements have been shown to increase with increasing levels of protein, the procedure used is similar to that used to induce amino acid imbalances. Therefore, there is also need for reinvestigation of the influence of protein level on amino acid requirements. An investigation of this subject, using wheat gluten as the source of protein, will shortly be reported.[3]

Finally it seems clear that the magnitude of the growth depression caused by adding to a diet a mixture of amino acids that creates an imbalance depends upon the rate of gain supported by the original diet. When the diet provides all of the indispensable amino acids in quantities that exceed the

[2] Kumta and Harper, unpublished results.
[3] Munaver and Harper, unpublished results.

requirements the diet may be thrown considerably out of balance without causing an adverse effect (fig. 1). This raises the question of the relationship between the amino acid balance of a protein and its nutritive value. Some proteins are so much out of balance (such proteins as zein, gelatin and hemoglobin which lack an amino acid), that they cannot satisfy the amino acid requirements of an animal under any circumstances. We do not, however, know at what degree of unbalance it becomes impossible to satisfy the requirements of an animal by increasing the level of protein in the diet, i.e., the stage at which amino acid supplementation becomes mandatory. This can be answered empirically by determining the effects on the growth rate of adding different levels of an amino acid mixture lacking a single acid to diets containing various levels of protein. The results of such a study could be used to calculate the point at which we pass from the stage of a simple deficiency to that of an amino acid imbalance.

SUMMARY

A protein lacking tryptophan (gelatin) or an amino acid mixture lacking threonine has been used to create amino acid imbalances involving tryptophan or threonine in diets containing casein supplemented with methionine.

The magnitude of the growth depression caused by the addition of gelatin increased as the level of gelatin was increased.

The magnitude of the growth depression caused by the amino acid mixture lacking threonine was greatest when the diet contained 6% of casein and was less if the level of casein in the diet was lower or higher.

The magnitude of the growth depression caused by a constant amount of the amino acid mixture lacking threonine was shown to be related to the rate of growth supported by the original diet. However, the increase in the level of threonine required to prevent the growth depression appeared to be unaffected by the level of protein in the original diet.

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THE EFFECT OF RADIATION STERILIZATION ON THE NUTRITIVE VALUE OF FOODS

IV. ON THE AMINO ACID COMPOSITION OF GARDEN PEAS AND LIMA BEANS [1]

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The use of irradiation sterilization for the preservation of foods may simplify certain food storage and shipping problems. Irradiation can destroy microorganisms in the food but may also affect the acceptability and the nutritive value of the food. The purpose of this paper is to report the changes in the amino acid composition of lima beans *(Phaseolus lunatus)* and garden peas *(Pisum sativum)* brought about by irradiation.

The fact that amino acids may be destroyed by ionizing radiation has been known for a number of years (Johnson, '57). The mechanisms of destruction have been reported to include deamination, decarboxylation, liberation of hydrogen sulfide from sulfhydryl groups of disulfide bonds (Proctor and Bhatia, '53), rupture of the benzene ring (Proctor and Bhatia, '53), opening of the indole ring of tryptophan (Jayson, Schales and Weiss, '54), masking of functional groups by cross-linking, etc.

[1] These studies were supported in part under contract no. DA-49-007-MD-544, with the Office of the Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of the Army.

[2] Irradiation was carried out by Phillips Petroleum Company, Arco, Idaho. 1 rad = 100 ergs per gram of tissue; gamma irradiation was used to irradiate the foods.

In the present study, hydrolysates of non-irradiated, 2.8-million-rad-irradiated,[2] and 9.3-million-rad-irradiated [2] lima beans and garden peas have been analyzed by the column chromatographic method of Moore and Stein ('48) and of Stein ('53).

EXPERIMENTAL

Preparation of samples

Frozen lima beans and green peas were purchased from local food stores. The samples were unpacked, mixed, and repacked in no. 2 cans, refrozen and stored at —20°C. One third of these samples was treated with 2.8 million rads γ-irradiation, the second third with 9.3 million rads γ-irradiation, and the remaining third was not irradiated.

Prior to hydrolysis, three kinds of samples were prepared from non-irradiated and from irradiated peas and beans: (a) samples of dry powdered peas or beans; (b) performic acid-oxidized samples; (c) enzyme-digested samples for the removal of carbohydrates prior to hydrolysis.

Dry powdered samples. Peas or beans were crushed and held in a drying oven at 45°C under 29 inches of vacuum until a constant weight was reached. They were then ground in a Wiley micromill, and held in a vacuum desiccator in the cold-room. Dustin, Czajkowska, Moore and Bigwood ('53) have shown that, except for cystine, methionine, and tryptophan, amino acids are not lost during hydrolysis in the presence of large amounts of carbohydrate under the dilute conditions of acid hydrolysis used in this study.

Performic acid-oxidized samples. The determination of sulfur-containing amino acids following performic acid oxidation has been reported (Block and Weiss, '56). Samples of dry powdered beans and peas containing 20 mg of protein were mixed with 9 ml of formic acid (88% w/w) and 1 ml of H_2O_2 (30% w/w). The mixtures were stirred gently for 30 minutes at room temperature. At the end of the reaction time, most of the reagent was removed under reduced pressure

at 40°C, and the resulting syrupy residues were immediately suspended in 6N HCl for hydrolysis.

Enzyme-digested samples. Since the hydrolysis of proteins embedded in carbohydrate matrix, such as in the tissue structure of green peas and lima beans, results in the destruction of methionine and cystine (Dustin et al., '53), and since it was found that the performic acid oxidation of methionine in beans or peas resulted in the formation of methionine sulfone which was eluted from the column with the serine peak and thus could not be quantitatively recovered, the removal of carbohydrates from dry powdered beans or peas prior to hydrolysis was carried out by an enzymatic method.

One-half gram of dry powdered sample was weighed, suspended in 5 ml of water, and gelatinized by cooking at 90 to 95°C. (5 gm of centrifuged saliva may be used to replace 0.5 gm of malt diastase.) The suspension was incubated at 37°C for 48 hours under toluene. At the end of incubation, the whole suspension was transferred to a dialyzing bag and dialyzed against three portions of 500 ml distilled water for 72 hours at about 4°C. At the end of the third dialyzing period, the contents of the dialysis bag were hydrolyzed. Since the dialysate gave a positive reaction to ninhydrin and a negative biuret color, it was condensed to about 30 ml at 40°C by the use of a (Schaar) rotating evaporator, combined with the hydrolysate, and put on the column. An enzyme blank was determined, and its amino acid composition was used as a basis for correcting the results of the analysis of the hydrolyzed peas and beans.

Hydrolysis of samples

Each sample was placed in a sealed vacuum tube with 3000 times as much 6 N HCl as there was protein in the sample, and the sample was hydrolyzed under vacuum in a 110°C oven for 24 hours. The hydrolysates were concentrated at 45°C under reduced pressure until nearly dry. Ten milliliters of water were added and the hydrolysates were concentrated again to near dryness. They were then made up to volume with pH 3.4 buffer.

Tryptophan was determined microbiologically by the Miller and Ruttinger ('50) procedure following hydrolysis by autoclaving with 6 N $Ba(OH)_2$ for 10 hours at 15 lbs. pressure.

Chromatography of hydrolysates

Chromatography of hydrolysates was carried out as described by Moore and Stein ('48) and Stein ('53). The acidic and neutral amino acids were determined on a 100 cm Dowex 50 column, and the basic amino acids were determined on a 15 cm column. About 2.5 mg of protein were used for each determination. After the development of color, each fraction of effluent was read in a Beckman DU spectrophotometer with a 1 cm cell at 570 mu and 0.03 mm slit width. Proline fractions read at 440 mu. Optical densities over 0.5 were redetermined by dilution.

Attempted determination of methionine as its oxidized products

Food samples were oxidized with performic acid to convert methionine to sulfone because this compound has been assumed stable during hydrolysis in the presence of carbohydrates (see Block and Weiss, '56). It was found that not only methionine, but also methionine sulfoxide and methionine sulfone were variably destroyed during hydrolysis (from 22 to 87%) in the presence of 200 times the methionine as starch with 1000 times its weight of 6 N HCl for 24 hours. When methionine was oxidized with either performic acid or H_2O_2, little sulfoxide was formed, the oxidation proceeding to the sulfone. The sulfone emerged from the column along with the serine peak. Methionine sulfoxide, when studied, was found to emerge about 20 ml ahead of the sulfone and just ahead of, or mixed with, the aspartic acid peak. Thus, it appeared that the accurate determination of methionine as sulfoxide or sulfone was not possible, both because of variable destruction in the presence of carbohydrate and because of overlapping peaks. For this reason, studies were carried out on the removal of carbohydrates from the samples prior to hydrolysis.

Since the time this work was done, Bidmead and Ley ('58) have shown that if the oxidation is carried out at — 10°C and the performic acid is then removed by freeze-drying, no loss of methionine occurs and quantitative recovery as the sulfone can be obtained.

The removal of carbohydrates from the sample prior to hydrolysis

The variable losses of amino acids during hydrolysis in the presence of carbohydrates have made quantitative amino acid analysis of cereal proteins difficult. Many organic chemical methods (see Block and Weiss, '56) have been suggested to separate the carbohydrates from the proteins, but the enzymatic method is more specific and appeared to us to be more promising. Block and Bolling ('51) reported that a large portion of the starch in cereal grains may be removed by the use of amylases present in human saliva. It was found in this study that alpha and beta amylases alone cannot digest legume carbohydrates satisfactorily without the help of pectinase.

The removal of carbohydrates prior to hydrolysis by the use of amylase and pectinase resulted in an increase of 35% in the methionine obtainable from pea proteins, and an increase of 25% from lima bean proteins. A cystine peak was shown in each determination; however, it was low, spread out and with a dent on the top, and since the cystine peak of the enzyme blank (usually 0.5 gm of pectinase and 5 gm of saliva) was not clear, cystine was determined in terms of cysteic acid (Tsien and Johnson, '59).

The total lysine in green peas was not determinable unless the carbohydrates were removed prior to hydrolysis. In addition to methionine and lysine, the following amino acids in pea proteins also were increased by the indicated percentages: serine 16%, threonine 27%, leucine 8%, and phenylalanine 5%. For lima beans, the removal of carbohydrates by enzymes did not make as much difference.

The commercially produced pectinases (usually consisting of two or more pectic enzymes) were found by Carpenter and Walsh ('32) to contain proteolytic enzymes. In this study also, the dialysates gave a slightly bluish color in the biuret test. Short-chain peptides present in the dialysate, as shown by paper chromatography, accounted for a decrease of certain amino acids in the process of removing carbohydrates. This type of loss is not involved in any of the values given in the table. The elimination or inhibition of proteolytic enzymes from the pectinases would help the problem of removing carbohydrates from samples prior to hydrolysis.

TABLE 1

The amino acid composition of saliva and enzymes
(Calculated as grams per 100 gm of crude protein, N × 6.25)

AMINO ACID	HUMAN SALIVA (3 DETERMINATIONS)	PECTINASE [1] (3 DETERMINATIONS)	MALT DIASTASE [1] 2 LONG COLUMNS ONE SHORT COLUMN
Aspartic acid	7.58 ± 0.07 [2]	9.58 ± 0.04 [2]	2.59
Threonine	4.30 ± 0.09	3.52 ± 0.11	0.34
Serine	5.42 ± 0.11	4.16 ± 0.16	0.33
Glutamic acid	7.80 ± 0.04	9.80 ± 0.03	1.41
Proline	2.31 ± 0.06	0.90 ± 0.05	7.18
Glycine	7.39 ± 0.06	4.27 ± 0.07	9.66
Alanine	1.50 ± 0.09	3.92 ± 0.02	5.18
Cystine and cysteine [3]			0.40
Valine	6.00 ± 0.03	1.41 ± 0.02	2.61
Methionine	0.91 ± 0.09	0.78 ± 0.14	0.37
Isoleucine	5.00 ± 0.05	1.86 ± 0.06	1.69
Leucine	6.39 ± 0.05	2.41 ± 0.05	2.91
Tryosine	2.10 ± 0.02	2.96 ± 0.04	0.73
Phenylalanine	1.01 ± 0.03	3.06 ± 0.02	3.10
Tryptophan [4]			
Histidine	1.81 ± 0.16	2.21 ± 0.19	0.51
Lysine	7.79 ± 0.17	5.50 ± 0.05	2.78
Arginine	2.89 ± 0.09	3.00 ± 0.14	6.23
Ammonia	1.81 ± 0.18	7.22 ± 0.27	4.45
Total	72.01	66.56	52.47
% of crude proteins	0.3325	7.125	26.28

[1] Commercial products from Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Standard error.
[3] Cystine and cysteine were determined as cysteic acid.
[4] Tryptophan was determined microbiologically.

The amino acid composition of human saliva, pectinase, and malt diastase are summarized in table 1. The amino acid composition of human saliva from the same individual was found to be constant.

Effect of irradiation on green peas and lima beans

The freshly irradiated peas were not different in odor or appearance from the non-irradiated peas, but after drying and grinding, the 2.8-million-rad-irradiated peas appeared yellowish brown and the 9.3-million-rad-irradiated peas were deep

TABLE 2

The effect of irradiation on amino acid composition of peas

(Calculated as grams per 100 gm of crude protein, N × 6.25)

AMINO ACID	IRRADIATION DOSAGE IN MILLION RAD		
	0 (4 determinations)	2.8 (4 determinations)	9.3 (4 determinations)
Aspartic acid	8.47 ± 0.08 [1]	8.54 ± 0.06 [1]	8.46 ± 0.04 [1]
Threonine	4.26 ± 0.19 [2]	4.31 ± 0.20 [2]	4.30 ± 0.24 [2]
Serine	4.74 ± 0.07 [2]	4.58 ± 0.16 [2]	4.60 ± 0.11 [2]
Glutamic acid	13.10 ± 0.12	12.90 ± 0.13	13.32 ± 0.17
Proline	3.01 ± 0.10	3.09 ± 0.11	3.05 ± 0.18
Glycine	3.61 ± 0.11	3.59 ± 0.09	3.69 ± 0.06
Alanine	3.90 ± 0.05	3.60 ± 0.04	3.78 ± 0.08
Cystine and cysteine [3]	0.70 ± 0.04	0.71 ± 0.06	0.63 ± 0.06
Valine	4.82 ± 0.02	4.85 ± 0.04	4.76 ± 0.04
Methionine	1.21 ± 0.14 [2]	1.16 ± 0.19 [2]	1.17 ± 0.15 [2]
Isoleucine	3.27 ± 0.09	3.19 ± 0.07	3.26 ± 0.03
Leucine	5.81 ± 0.07 [2]	5.79 ± 0.02 [2]	5.78 ± 0.04 [2]
Tyrosine	2.75 ± 0.03	2.71 ± 0.04	2.76 ± 0.04
Phenylalanine	5.14 ± 0.06 [2]	5.11 ± 0.09 [2]	5.09 ± 0.5 [2]
Tryptophan [4]	1.52 ± 0.02	1.54 ± 0.02	1.59 ± 0.03
Histidine	1.46 ± 0.18	1.47 ± 0.16	1.53 ± 0.19
Lysine	8.60 ± 0.06 [2]	7.10 ± 0.10 [2]	5.60 ± 0.08 [2]
Arginine	13.46 ± 0.16	11.86 ± 0.14	9.60 ± 0.09
Ammonia	2.08 ± 0.10	2.03 ± 0.14	2.05 ± 0.16
Total	91.91	88.13	85.02

[1] Standard error.

[2] Carbohydrates in the sample were digested by enzymes and removed prior to hydrolysis.

[3] Cystine and cysteine were determined as cysteic acid.

[4] Tryptophan was determined microbiologically.

brown. The irradiated lima beans were somewhat bleached and had a different odor than the raw beans; the dry powder of the irradiated beans also appeared slightly brownish. The non-irradiated, dry powdered peas and beans were a light green color.

Effect of irradiation on the amino acid composition of peas and lima beans

In both legumes, arginine and lysine were markedly destroyed. Statistical analysis (Snedecor, '50) showed that in both legumes, lysine and arginine levels were significantly

TABLE 3

The effect of irradiation on amino acid composition of lima beans

(Calculated as grams per 100 gm of crude protein, N × 6.25)

AMINO ACID	IRRADIATION DOSAGE IN MILLION RAD		
	0 (4 determinations)	2.8 (4 determinations)	9.3 (4 determinations)
Aspartic acid	10.74 ± 0.14 [1]	10.76 ± 0.09 [1]	11.23 ± 0.11 [1]
Threonine	4.04 ± 0.09	4.00 ± 0.06	3.95 ± 0.05
Serine	6.25 ± 0.12	6.22 ± 0.04	6.30 ± 0.10
Glutamic acid	12.88 ± 0.14	13.01 ± 0.13	12.99 ± 0.13
Proline	1.50 ± 0.13	1.56 ± 0.09	1.55 ± 0.14
Glycine	4.00 ± 0.08	3.98 ± 0.03	4.10 ± 0.03
Alanine	3.80 ± 0.09	4.03 ± 0.04	4.02 ± 0.05
Cystine and cysteine [2]	1.10 ± 0.05	1.06 ± 0.06	1.07 ± 0.09
Valine	5.31 ± 0.02	5.48 ± 0.04	5.40 ± 0.02
Methionine	1.56 ± 0.12 [3]	1.57 ± 0.14 [3]	1.49 ± 0.16 [3]
Isoleucine	5.14 ± 0.03	5.04 ± 0.06	5.04 ± 0.04
Leucine	8.22 ± 0.02	8.06 ± 0.04	8.10 ± 0.04
Tyrosine	3.00 ± 0.07	2.98 ± 0.06	2.96 ± 0.03
Phenylalanine	6.22 ± 0.04	6.28 ± 0.09	6.32 ± 0.07
Tryptophan [4]	2.60 ± 0.03	2.70 ± 0.02	2.60 ± 0.03
Histidine	3.21 ± 0.17	2.93 ± 0.14	3.10 ± 0.09
Lysine	10.44 ± 0.05	9.21 ± 0.09	5.83 ± 0.07
Arginine	7.57 ± 0.10	6.05 ± 0.08	4.23 ± 0.06
Ammonia	2.21 ± 0.10	2.20 ± 0.12	2.17 ± 0.15
Total	99.79	98.12	93.45

[1] Standard error.

[2] Cystine and cysteine were determined as cysteic acid.

[3] Carbohydrates in the sample were digested by enzymes and removed prior to hydrolysis.

[4] Tryptophan was determined microbiologically.

different (at the 1% level) for each treatment. The amount of destruction of both basic amino acids was closely related to the level of irradiation. No other amino acid was significantly affected during irradiation. The data are summarized and tabulated in table 2 for green peas and in table 3 for lima beans.

SUMMARY

In both peas and beans, lysine and arginine were extensively destroyed by irradiation, the amount of destruction increasing with increasing irradiation dosages.

The use of enzymes to digest and remove carbohydrates from the food prior to hydrolysis appeared to be the best method of preparing these samples for acid hydrolysis.

The determination of methionine in terms of methionine sulfoxide or sulfone was unsatisfactory and needs further investigation before it can be applied to foods high in carbohydrate.

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GROWTH RATE AND LYSINE REQUIREMENT OF THE CHICK[1]

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The elucidation of amino acid requirements for growth frequently necessitates the use of highly purified diets. With such diets, growth is often suboptimal, even though the diet appears to contain all nutrients, including amino acids, required for optimal growth (Russell and Taylor, '48; Nasset, '57). Observations of this nature have caused the validity of the range of requirements thus established to be questioned, since the rate of growth of an animal might influence the requirement for essential amino acids.

When the methionine, tryptophan, and lysine requirements of chicks selected for fast and slow growth (presumably genetic differences) from a large population at two weeks of age were studied by Griminger ('55), no significant differences in requirements were found between fast- and slow-growing chicks. While the chicks chosen for slow growth gained less in absolute weight during their two-week experimental periods than those chosen for fast growth, their rates of growth, whether calculated as per cent of starting weight or of mean weight, or by the use of Brody's ('45) formula for instantaneous rate of growth per day, were actually

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[2] A part of the experimentation was carried out by one of the authors (P. G.) at the Department of Poultry Husbandry, University of Nebraska, Lincoln, Nebraska.

greater than those of the faster growing birds. It was evident, however, that while growth differed, the requirements, as a percentage of the diet, for the amino acids under investigation remained the same for the two groups.

Edwards et al. ('56), comparing diets containing wheat gluten or sesame meal as the protein source, obtained optimum chick growth with the respective diets at different levels of lysine. Their studies indicated a higher requirement for the chicks receiving the sesame meal, which also grew at a much faster rate than those receiving the wheat gluten diet. These authors interpreted this to mean that the lysine requirement of the chick is related to the rate of growth.

In the present paper evidence will be presented to the effect that rate of growth does not, *per se,* influence the lysine requirement (expressed as a percentage of the diet) of the growing chick. An explanation of the findings of Edwards et al., which are seemingly at variance with this hypothesis, will be suggested.

EXPERIMENTAL

Lysine was used as the limiting amino acid, since it is the amino acid concentrated to the greatest degree in muscle and organ tissue, and the lysine requirement would therefore appear most likely to be influenced by the rate of growth, if such an influence could be demonstrated (Almquist, '52). The lysine requirement of growing chicks was determined under 4 different conditions: (1) with chicks from one population selected for fast and slow growth, (2) with and without a non-nutritive growth depressant, (3) with chicks from different breeds growing at different rates, and (4) with and without the chicks having been inoculated with the virus of bronchitis.

To fulfill condition (1), female "Indian River" broiler-type chicks obtained from a commercial hatchery were raised on a practical diet adequate in lysine. At two weeks of age, 160 fast- and an equal number of slow-growing chicks were selected from a population containing over 600 individuals,

those growing at an intermediate rate being rejected. After distributing the chicks by weight into 20 pens of 8 chicks for each growth group, two pens from each growth group were assigned to each level of lysine fed with the basal diet no. 1 shown in table 1. The chicks were kept on the experimental

TABLE 1

Composition of experimental rations

INGREDIENT	NO. 1	NO. 2
	%	%
Sesame meal	45.00	39.00
Cerelose	44.06	44.46
Corn oil, refined	2.00	1.00
Alfalfa meal,dehydrated	3.00	—
Alfalfa meal, sun-cured	—	2.50
Fish solubles	—	2.50
Distillers dried solubles	—	2.50
Dried whey	—	2.50
Salt mixture [1]	5.34	5.34
Choline-Cl	0.20	0.20
DL-Methionine	0.15	—
Vitamin A conc. (3000 I.U./gm)	0.15	—
Vitamin D conc. (1500 I.C.U./gm)	0.10	—
Total [2]	100.00	100.00

[1] See Griminger et al. ('56a), except that in ration no. 1 ferrous citrate was replaced by an equal amount, by weight, of ferrous gluconate.

[2] Plus the following supplements (milligrams per kilogram diet): Ration no. 1: thiamine·HCl, 2; riboflavin, 3; niacin, 25; calcium pantothenate, 10; pyridoxine·HCl, 3; folic acid, 0.5; biotin, 0.1; cyanocobalamin, 0.01; α-tocopheryl acetate, 20; ascorbic acid, 100; menadione sodium bisulfite complex, 1; procaine penicillin, 10. Ration no. 2: thiamine·HCl, 100; riboflavin, 16; niacin, 100; pyridoxine·HCl, 6; folic acid, 4; biotin, 0.6; cyanocobalamin, 0.02; inositol, 100; para-aminobenzoic aeid, 2; ascorbic acid, 250; menadione, 5; α-tocopheryl acetate, 20; procaine penicillin, 11; 10,000 I.U. of vitamin A (acetate) and 600 I.C.U. of vitamin D_3.

diets for 14 days, and their weights and feed consumption recorded weekly. In this, as in all other experiments reported, the chicks were raised on wire floors in electrically heated battery brooders, and feed and water were supplied ad libitum.

The investigations of the influence of slow growth resulting from the addition of a growth depressant (saponin) and by the use of breeds growing at different rates [conditions (2) and (3) above] were carried out within the framework of one experiment. At 13 days of age 80 male and an equal number of female chicks were selected from a large population of crossbred chicks (New Hampshire ♂ × Columbian ♀) by discarding the individuals at the extremes of the body weight range. Similarly, an equal number of birds was selected from a group of chicks originating from an inbred line of S. C. White Leghorns known for slow growth. The chicks from each breed were assigned by weight to 20 pens of 8 chicks (4 ♂ and 4 ♀), two pens per breed being assigned to each level of lysine supplementation.

The experimental period and the basal diet were the same as in the previous test, except that one pen of crossbred chicks on each level of lysine received 0.6% Quillaja saponin (tannin-free) in their diet replacing an equal amount, by weight, of cerelose.

In the last experiment, 240 female crossbred chicks (N. H. ♂ × Col. ♀) were distributed by weight into 24 pens of 10 chicks each at one week of age. The composition of the experimental diet is shown in table 1 (diet no. 2). The chicks in 12 pens were inoculated with bronchitis virus by introducing a suspension of the virus intratracheally, while the other pens, housed in a different location, served as non-inoculated controls. They were then fed 6 graded levels of lysine in their diet, so that duplicate pens of each group (inoculated and control) received identical rations. The experiment was continued for three weeks, and weights and feed consumption were recorded at weekly intervals.

In all experiments, lysine was added in form of 95% L-lysine HCl, with due allowance for the 5% diluent and the HCl. On the basis of microbiological assay of the protein sources, basal diet 1 contained 0.53% of lysine, while basal diet 2 was found to contain 0.63% of lysine.

RESULTS AND DISCUSSION

For the purpose of clear presentation, one criterion of lysine sufficiency in each experiment is presented in graphic form, while the others are tabulated. Gains and rates of growth were calculated for each chick, using, for the latter, Brody's ('45) formula for instantaneous percentage rate of growth per day (table 2, footnote 3), which is based on the growth-curve equation $W = Ae^{kt}$. For the graphs, the logarithms of the dose (% lysine of the diet) were plotted against the respective criterion of response (growth, gains, or rate of growth), as proposed by Almquist ('53). Where replicate pens were used, one set of data for each experiment is given for each pen separately in order to illustrate the magnitude of variation between pens. The graphs were constructed on the basis of regression lines of individual weights, gains, or growth rates respectively, for the ascending part of the response curve and the arithmetic mean of all values for the horizontal part of the response curve.

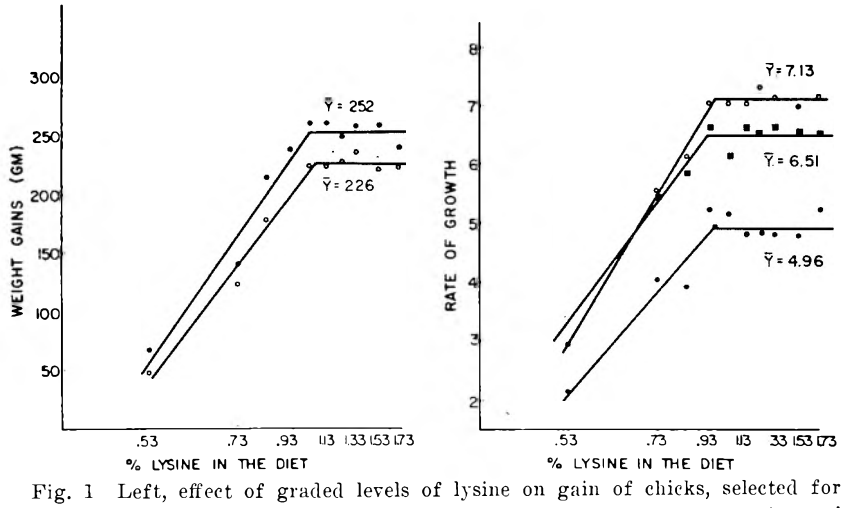

Fig. 1 Left, effect of graded levels of lysine on gain of chicks, selected for fast (●) or slow (○) growth at 13 days, during subsequent 14-day experimental period. Right, effect of graded levels of lysine on the rate of growth (table 2, footnote 3) of chicks from a slow-growing inbred Leghorn line (■), of crossbred chicks (○), and of crossbreds receiving 0.6% Quillaja saponin as a growth depressant (●), during 14-day experimental period.

It appears from figure 1 and table 2 that optimum gains, weights, and growth rates for both groups in the first experiment were obtained at the 1.03% level of lysine. Optimum

TABLE 2

Growth and feed efficiency data of fast-growing (lots 1–10) and slow-growing (lots 11–20) chicks receiving graded levels of lysine

LOT [1]	TOTAL LYSINE	AV. WEIGHT [2] AT 27 DAYS OF AGE			AV. RATE OF GROWTH (K) [3] DURING 14-DAY EXPTL. PERIOD ($R_1 + R_2$)	GAIN/FEED ($R_1 + R_2$)
		R_1	R_2	Av.		
	%		gm			
1	0.53	232	243	238	2.4	0.24
2	0.73	311	310	311	4.3	0.37
3	0.83	377	389	383	5.8	0.44
4	0.93	397	415	406	6.2	0.49
5	1.03	432	426	429	6.6	0.52
6	1.13	431	427	429	6.7	0.52
7	1.23	410	424	417	6.5	0.53
8	1.33	432	419	426	6.6	0.53
9	1.53	431	420	426	6.6	0.54
10	1.73	410	405	408	6.3	0.51
11	0.53	180	177	179	2.2	0.23
12	0.73	261	248	254	4.7	0.38
13	0.83	316	302	309	6.1	0.45
14	0.93	342	315	329	6.5	0.48
15	1.03	344	362	353	7.1	0.51
16	1.13	344	367	355	7.1	0.55
17	1.23	372	345	358	7.2	0.53
18	1.33	371	361	366	7.3	0.55
19	1.53	350	354	352	7.0	0.54
20	1.73	348	358	353	7.1	0.55

[1] Two pens ($R_1 + R_2$) of 8 chicks each per lot.

[2] Average weights of chicks at 14 days of age (start of experimental period): fast-growing lots, 168 to 171 gm (range of individual chicks, 159 to 198): slow-growing lots, 130 to 133 gm (range of individual chicks, 113 to 141).

[3] Instantaneous percentage rate of growth per day $K = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \times 100$ (Brody, '45), where W_2 and W_1 represent the weight of chicks at t_2 and t_1 days respectively.

feed conversion for the fast-growing chicks was reached at the same level, but not until the 1.13% level for the slow-growing chicks.

Scrutiny of the growth rates of this experiment reveals that the terms "fast-" and "slow-growing" for the two groups of chicks are relative indeed. Homeyer and Pauls ('54) obtained correlations greater than 0.5 between zero to two-week gains and two to 4-week gains on two large groups of chicks. Nevertheless in the present results, when broken down into growth groups at two weeks of age, a clear tendency can be observed for the smaller chicks to add more weight on a percentage basis than the larger chicks. Thus, the numerical values of growth rates are found to be greater for the smaller chicks. Consequently, there should be a smaller maintenance requirement; indeed, table 2 indicates a tendency for the gain/feed ratios to be better for the smaller chicks.

Statistical analysis for this experiment shows that final growth data were significantly influenced by association with a certain growth group or by lysine level, but that no significant interaction exists between these two sources of variation. On this basis, and from inspection of the other criteria tabulated, it can be concluded that faster growth did not influence the lysine requirements for optimum growth. Even if fast growth is judged from the point of view of growth rate, thus considering the smaller chicks as faster growing, the above view need not be altered.

In the next phase an attempt was made to induce slower growth and lower growth rates by means of a known growth depressant. The growth-depressing properties of saponin have been discussed by Peterson ('50). It is not unlikely that decreased appetite and feed intake play a major role in this depression. Preliminary experiments with this diet had indicated that a significant growth depression could be obtained by the inclusion of 0.6% of saponin, both with lysine-deficient and with lysine-adequate diets. In addition, a slow-growing strain of Leghorns was included in this experiment. Figure 1 and table 3 show that while saponin reduced gains and growth rates to approximately two-thirds of normal, it did not alter the level of lysine at which optimum growth was registered. Similarly, the chicks from a highly inbred line of

TABLE 3

Response of crossbred chicks to graded levels of lysine in the presence and absence of a growth depressant (Quillaja saponin); response of slow growing inbred Leghorns to the same levels of lysine

LOT	TOTAL LYSINE	CROSSBREDS [1]						LEGHORNS [2]				
		Controls			Saponin							
		Av. weight [3]	Av. gain [4]	Gain/feed [4]	Av. weight [3]	Av. gain [4]	Gain/feed [4]	Av. weight [3]	R_1	Av. gain [4] R_2	Av.	Gain/feed [4] $(R_1 + R_2)$
	%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
1	0.53	264	90	0.27	237	62	0.22	157	64	55	59	0.25
2	0.73	380	205	0.40	308	134	0.35	208	109	114	112	0.37
3	0.83	413	238	0.44	306	132	0.37	225	124	131	127	0.42
4	0.93	470	295	0.50	365	191	0.47	248	151	149	150	0.47
5	1.03	468	294	0.52	362	188	0.47	234	131	142	136	0.47
6	1.13	472	297	0.54	343	172	0.45	246	148	151	150	0.47
7	1.23	488	314	0.54	347	173	0.49	244	144	149	147	0.47
8	1.33	472	299	0.55	346	171	0.45	248	150	153	151	0.49
9	1.53	465	291	0.52	348	171	0.46	246	141	155	148	0.46
10	1.73	475	301	0.54	365	191	0.48	246	147	148	148	0.48

[1] One pen of 4 males + 4 females per lot.

[2] Two pens of 4 males + 4 females per lot.

[3] At 27 days of age. Average weights of chicks at 13 days of age (start of experimental period): crossbred lots, 173 to 177 gm (range of individual chicks, 165 to 188): Leghorn lots, 94 to 98 gm (range of individual chicks, 79 to 116).

[4] During 14-day experimental period.

Leghorns registered optimum gains and growth rates within their genetic capabilities at the same level of lysine (0.93%). Optimum gain/feed ratios were also obtained at this lysine level except, perhaps, in the case of the control group. Feed efficiency values were lower for both the saponin and Leghorn groups as compared with the control group.

The authors are aware of existing evidence that breed differences may influence amino acid requirements (Hegsted et al., '41; McDonald, '57). Hegsted et al. explained their findings on the basis of a higher arginine and glycine require-

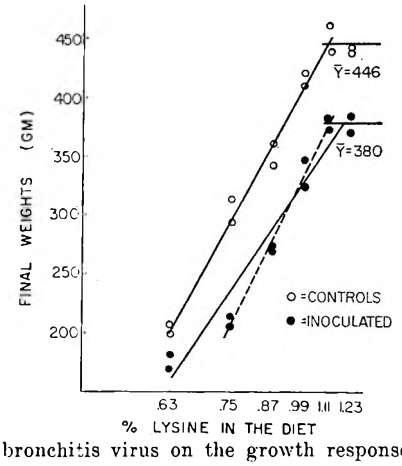

Fig. 2 Effect of bronchitis virus on the growth response of chicks at 4 weeks of age to graded levels of lysine. Each symbol represents one lot of 10 chicks. Broken line indicates regression line of growth of inoculated chicks excluding response to the unsupplemented (0.63% lysine) lots.

ment for fast-feathering breeds during the period of rapid feather development. McDonald's findings appear to be due to a specific metabolic block in sulfur amino acid metabolism in a certain breed (Australorps) rather than to a difference in growth rate between the breeds examined (McDonald, '58). Both of these studies are concerned with breed differences other than the rate of growth and therefore are not pertinent to the present investigation.

In the last experiment, using a specific virus as a growth depressant (Biester and Schwarte, '48), optimum growth was

obtained for both the infected and the control group at the 1.11% level of lysine (fig. 2 and table 4). Growth rates and gain/feed ratios followed the same pattern. As in the previous experiment, smaller gains coincided with lower rates of growth and gain/feed ratios.

The inoculated chicks receiving the basal diet grew better than would be expected from the responses noted for the

TABLE 4

Effect of bronchitis virus on the response of chicks to graded levels of lysine

LOT [1]	TOTAL LYSINE	GAIN [2]			AV. RATE OF GROWTH [3] ($R_1 + R_2$)	GAIN/FEED ($R_1 + R_2$)
		R_1	R_2	Av.		
	%	*gm*	*gm*	*gm*	*gm*	*gm*
			Controls			
1	0.63	106	114	110	3.6	0.27
2	0.75	221	202	211	5.6	0.35
3	0.87	251	268	260	6.3	0.40
4	0.99	326	316	321	7.1	0.44
5	1.11	370	346	358	7.5	0.47
6	1.23	347	349	348	7.4	0.47
			Bronchitis-inoculated			
1	0.63	77	87	82	3.0	0.24
2	0.75	122	116	119	3.9	0.29
3	0.87	182	179	180	5.1	0.35
4	0.99	234	255	245	6.1	0.42
5	1.11	288	280	284	6.6	0.45
6	1.23	277	291	284	6.7	0.43

[1] Two pens ($R_1 + R_2$) of 10 chicks each per lot.

[2] During 21-day experimental period. Average weights of lots at 7 days (start of experimental period), 93 gm. Range of individual chicks, 83 to 111.

[3] See table 2, footnote 3.

higher levels of lysine. When the basal level is omitted, the regression line through the increasing values (broken line in fig. 2) represents a much better fit; it must not be forgotten, however, that the log dose presentation of response to graded levels of amino acids does not necessarily have universal application. The good fit generally obtained when using this method does not mean that a good fit must be obtained under all circumstances. It is possible that in an infected population

growth depression will not be as great, percentagewise, at an extremely low level of lysine, as with a lysine-supplemented diet. The two lines of figure 2 will help to visualize this difference.

While the requirement for lysine appears to agree well within each experiment, there are marked differences between experiments with variations in the optimum lysine level ranging from 0.93 to 1.11%. Even when differences in location, time, and experimental conditions are taken into account, and although these data fall within the range of experimental values of other authors, the differences cannot be dismissed as meaningless. As different samples of sesame meal (and other ingredients) had been used in these experiments, it is possible that the lysine of sesame meal, evaluated by microbiological methods, might not always be available to the chick to the same extent (Edwards et al., '56).

The evidence available from these three experiments points overwhelmingly to the conclusion that differences in growth rate of chicks do not, *per se,* necessitate different dietary levels of lysine for optimum growth. These findings seem to support Almquist's ('47) view that the proportions of indispensable amino acids remain the same for any suboptimal rate of growth. Lower feed consumption would satisfy the lower absolute requirement for lysine of a slow-growing bird without changing the requirement of lysine as a percentage of the feed.

Edwards et al. ('56), determining the lysine requirement with two different diets, obtained a lower requirement with the wheat gluten diet supporting poorer growth. The cause of the poor growth with this diet, according to the authors, might have been a deficiency of amino acids other than lysine. The calculated amino acid composition of this diet seems to bear this out. It seems appropriate to quote a passage from Mitchell ('54): ". . . . for example, if lysine is the limiting amino acid for a food protein that contains only one-half of the amount of lysine in the standard, then for purposes of growth only, the utilization of all other essential amino acids

would be limited to 50 per cent of the amount present in the standard.'' The feeding of a poor diet, deficient in one or more of the essential amino acids, is, to a certain extent, similar to the feeding of a low-protein diet, as the amount of protein available for growth will be restricted by the most limiting amino acid. There is ample evidence that, in agreement with theoretical considerations, amino acid requirements at suboptimal levels of protein are lower than for optimal protein levels (Griminger et al., '56b; Bressani and Mertz, '58).

The lower growth rate and lower requirement found by Edwards et al. with the wheat gluten diet can both be explained as the result of the amino acid deficiency in the test diet, without a cause and effect relationship between growth rate and lysine requirement. Since the low growth rate appears to have been obtained as the result of the lack of ample amounts of balanced protein, the generalization regarding the effect of a low growth rate on amino acid requirement does not seem to be justified.

Mitchell and Beadles ('52) found that the protein percentage for maximum growth of rats was independent of caloric intake level within a considerable range, when the caloric density of the diet remained the same. Becker et al. ('54) found that a moderately depressed feed intake of pigs, due to dry feeding versus gruel feeding, and hence a decreased rate of gain, did not influence the protein requirement. Finally, Hutchinson ('57) observed that when lysine was the limiting factor in the basal rations, neither the level of feed intake on a restricted regime, nor the consequent difference in growth rate influenced the lysine requirement of the weanling rat. These reports add strength to the contentions presented here that rate of growth does not necessarily influence the requirement for essential amino acids when the requirement is expressed as a percentage of the diet.

SUMMARY

The influence of growth rate on the requirement of the young chick for lysine has been investigated. Variations in

the growth rate were achieved by selecting fast- and slow-growing individuals from one population, by adding a non-nutritive growth-depressant to the diet, by use of a fast- and a slow-growing group from two different breeds, and finally by intentionally infecting chicks with the virus of a respiratory disease (bronchitis).

In all cases, the difference in growth rate did not appear to influence the lysine requirement, when growth, gain, or the instantaneous rate of growth per day were used as criteria of lysine sufficiency.

ACKNOWLEDGMENT

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STUDIES ON THE ENHANCEMENT OF RADIOCALCIUM AND RADIOSTRONTIUM ABSORPTION BY LACTOSE IN THE RAT [1]

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The stimulating effect of dietary lactose on calcium absorption has been known for many years and has been shown to occur in rats (Bergeim, '26a), parathyroidectomized dogs (Greenwald and Gross, '29), dairy calves (Robinson et al., '29), chicks (Kline et al., '32) and children (Mills et al., '40). A recent paper by Lengemann et al. ('57) has re-emphasized the favorable effect of milk on calcium and strontium absorption and surveys past work. The metabolism of lactose has been reviewed by Duncan ('55) and Atkinson et al., ('57).

Although much attention has been given to the interrelationship between lactose and calcium metabolism, the mechanism by which lactose acts is still obscure. A popular explanation is based upon the fermentation of the poorly absorbed lactose by intestinal bacteria and the subsequent favorable effect of a lowered pH on calcium absorption. This theory has been supported by Bergeim ('26a), Robinson and Duncan ('31), Kline et al. ('32), and others, but has often been challenged, most recently by Fournier ('54) and Fournier et al. ('55) and by Wasserman et al. ('57). Fournier and associates had shown that many of the sugars that in-

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creased calcium retention were those that were attacked with difficulty by intestinal bacteria. The argument by Wasserman et al. ('57) was based on the observation that chicks, under short term conditions, did not respond to milk powder (containing lactose) while rats did show enhancement of calcium absorption. Both species have the potential for forming an intestinal bacterial population capable of rapidly fermenting lactose. Other theories are that lactose acts by diminishing the endogenous secretion of calcium into the intestine (French and Cowgill, '37), and that lactose functions as a "structural" entity to favor bone cell metabolism (Fournier, '54).

The present studies have been aimed at gaining basic information on the manner by which calcium and strontium pass across the gastrointestinal barrier, and how this movement is enhanced by lactose.

EXPERIMENTAL

Calcium absorption from the gastrointestinal tract was determined by measuring the uptake of Ca^{45} or Sr^{85} or both by bone after a single oral dose of a solution containing these radioisotopes. Both the present studies and previous reports (Wasserman et al., '56; Lengemann and Dobbins, '58) have shown the validity of employing this technique for studying calcium absorption under properly defined conditions.

The dosing solutions used in these studies contained 5 mg of calcium chloride, 5 μc of Ca^{45}, and 1 μc of Sr^{85} per milliliter plus lactose or other sugar to be tested at a level of 0.42 millimoles per mililiter, as indicated. Since radiostrontium behaves qualitatively the same as radiocalcium and is influenced by the same factors that affect calcium metabolism, the gamma-emitting radionuclide, Sr^{85}, was often employed as a more convenient indicator of calcium movement.

Young male albino rats [2] that weighed between 70 to 120 gm were used and unless otherwise noted, were fasted 24 hours

[2] Albino Farms, Red Bank, New Jersey.

prior to the experiment. For oral dosage, 2 ml of the test solution was given by gavage while the animal was under light ether anaesthesia and, unless otherwise indicated, these animals were killed 24 hours after dosage. In other experiments, 0.5 ml of the dosing solution was injected directly into the lumen of a ligated segment of the digestive tract while the animal was under deep ether anaesthesia; these animals were killed at 5 hours after dosage except those used for the time study. The femurs were removed and counted directly for Sr^{85} activity in a deep-well scintillation counter and, when necessary, ashed for the determination of Ca^{45} activity by the method described by Comar ('55). A study, presented later, showed that identical results were obtained whether the whole femur or the ashed sample was used. The results are expressed on the basis of "per cent of administered dose per femur."

In the vitamin D study, weanling albino male rats were kept on the Steenbock-Black ('25) rachitogenic diet for two weeks. Two days before isotope administration, 2000 I.U. of vitamin D (Viosterol) were given orally to one-half of the rats; the others received the vehicle alone (cottonseed oil). The animals were then handled as previously described.

RESULTS

The initial study was designed to indicate whether the lactose effect was dependent upon the type of calcium salt. The data, presented in table 1, show clearly that lactose did enhance the absorption of Ca^{45} and Sr^{85} ($p < 0.01$) and that this stimulation occurred whether the carrier calcium was present as the chloride, gluconate, lactate or acetate salt. This is in contrast with the data of Roberts and Christman ('42) who reported that lactose had no effect on the absorption of the lactate salt in the same species.

Table 2 presents data on the effect on lactose action of a strong chelating agent, sodium ethylenediaminetetraacetate (NaEDTA). Group II as compared with group I showed

TABLE 1

The influence of lactose and various calcium salts on the accumulation of orally administered Ca^{45} and Sr^{85} in rat femurs [1]

CALCIUM SALT	CA^{45} UPTAKE BY FEMUR		SR^{85} UPTAKE BY FEMUR		PERCENT CHANGE IN ABSORPTION WITH LACTOSE	
	No lactose	Lactose	No lactose	Lactose	Ca^{45}	Sr^{85}
	% dose/femur	*% dose/femur*	*% dose/femur*	*% dose/femur*	%	%
Calcium chloride	2.3 ± 0.3	3.6 ± 0.1	1.4 ± 0.3	2.8 ± 0.1	+ 57	+ 100
Calcium gluconate	2.1 ± 0.1	3.3 ± 0.2	1.2 ± 0.1	2.1 ± 0.2	+ 57	+ 75
Calcium lactate	1.9 ± 0.2	3.6 ± 0.2	1.1 ± 0.1	2.4 ± 0.2	+ 89	+ 118
Calcium acetate	2.0 ± 0.1	3.9 ± 0.1	1.1 ± 0.1	3.0 ± 0.2	+ 95	+ 173

[1] Each value represents mean ± standard error of the mean; 6 animals per group; each oral dose contained 3.61 mg Ca and 0.84 millimoles lactose; the experimental time was 24 hrs.

the usual enhancing effect of lactose on strontium absorption. Group III *vs.* group I demonstrated that NaEDTA itself had little or no effect on the amount of ingested Sr^{85} that reached the bone. Groups IV, V and VI showed that 0.16 millimoles of EDTA completely suppressed the lactose effect, whereas 0.04 millimoles of EDTA did not change the lactose effect.

To determine whether EDTA had any effect on movement of calcium between blood and skeleton, the animals in groups VII and VIII received intraperitoneal injections of Sr^{85} and carrier calcium with and without EDTA. It can be noted

TABLE 2

Influence of sodium ethylenediaminetetraacetate (NaEDTA) on the action of lactose in stimulating Sr^{85} absorption [1]

GROUP	IN DOSING SOLUTION			METHOD OF ADMINISTRATION	SR^{85} UPTAKE BY FEMUR	PERCENT CHANGE
	Ca^{++}	Lactose	NaEDTA			
	millimoles				*% dose/femur*	
I	0.042	0	0	Oral	1.5 ± 0.1	0
II	0.042	0.42	0	Oral	3.1 ± 0.2	+ 107
III	0.042	0	0.16	Oral	1.2 ± 0.1	− 20
IV	0.042	0.42	0.16	Oral	1.4 ± 0.2	− 7
V	0.042	0.42	0.08	Oral	2.2 ± 0.2	+ 47
VI	0.042	0.42	0.04	Oral	3.3 ± 0.1	+ 120
VII	0.021	0	0	Intraperitoneal	3.9 ± 0.1	—
VIII	0.021	0	0.08	Intraperitoneal	4.0 ± 0.2	—

[1] Values represent mean ± standard error of the mean; 6 rats per group.

that there was no effect of EDTA in these groups. It can therefore be inferred that the results of group III *vs.* group I can validly be interpreted to mean that NaEDTA did not alter the gastrointestinal absorption of the Sr^{85}.

The absorption of Sr^{85} was determined as a function of time after oral administration. From figure 1, it may be seen that lactose exerted its effect quite rapidly. By two hours the femurs of the lactose-treated rats contained more Sr^{85} ($p < 0.05$) than did those of the control animals. At 4 hours the uptake had leveled off in both groups with the lactose causing a doubling of the bone content. As part of the same study, the stomachs of the rats were also excised to deter-

mine the influence of lactose on gastric emptying time. The presence of lactose resulted in a decreased rate of removal of Sr^{85} from the stomach. At 30 minutes, 20% of the Sr^{85} dose was left in the stomach of the lactose-treated rats as compared with about 2% in the controls; this would mean that 80 and 98% of the activity in these respective groups had left the stomach. The difference between these values could not alone explain the enhancing effect of lactose on calcium absorption.

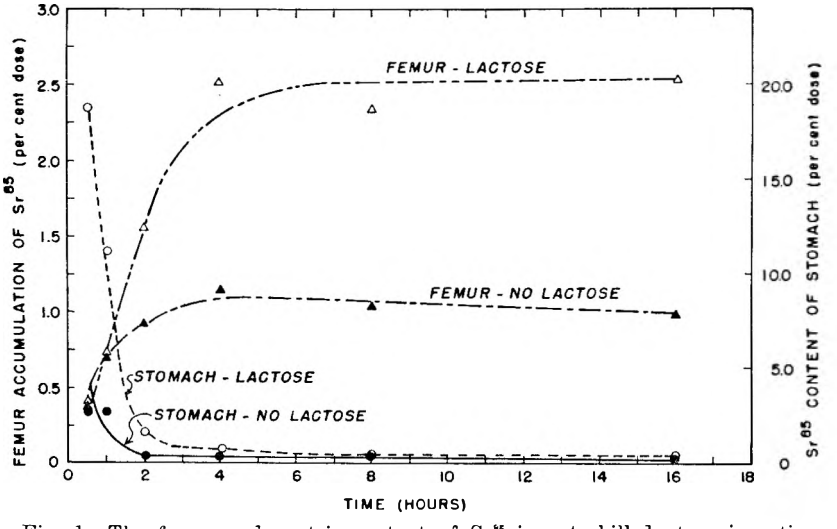

Fig. 1 The femur and gastric content of Sr^{85} in rats killed at various times after an oral administration of activity with or without lactose.

It was of interest to examine the effect of lactose on the absorption of Sr^{85} from different segments of the gastrointestinal tract. The intestinal segments are described as follows: duodenal segment — the first three to 4 inches of intestine distal to the pyloric sphincter; jejunal segment — three to 4 inches of the mid-portion of the small intestine; the ileal segment — the last three to 4 inches of the small intestine proximal to the ileo-cecal junction. The data are presented in table 3. Little absorption of Sr^{85} occurred from the stomach either in the presence or absence of lactose. The

greatest Sr^{85} absorption occurred from the duodenal and jejunal segments; absorption from the ileal segment was substantially less. All of the various segments of the small intestine showed a significant lactose effect ($p < 0.01$). Lactose stimulated Sr^{85} absorption from the upper segments by a factor of about 1.3 to 1.4, but most striking was the 4-fold increase in absorption from the ileal segment.

TABLE 3

The accumulation of Sr^{85} in rat femur after injection into various segments of the gastrointestinal tract [1]

GASTROINTESTINAL SEGMENT	TREATMENT				PERCENT INCREASE IN SR^{85}
	No lactose		lactose		
	% dose Sr^{85}/femur		*% dose Sr^{85}/femur*		
Stomach	0.08 ± 0.02	(6)	0.05 ± 0.01	(5)	no change
Duodenum	2.4 ± 0.1	(12)	3.4 ± 0.2	(11)	42
Jejunum	2.6 ± 0.2	(6)	3.4 ± 0.3	(6)	31
Ileum	0.55 ± 0.04	(6)	2.3 ± 0.2	(6)	320

[1] Values represent mean ± standard error of mean; dose contained 2.5 mg $CaCl_2$ and 0.21 millimoles of lactose in 0.5 ml solution; no. of animals per group given within parentheses; experimental time was 5 hr.

Since the ileum showed this marked enhancement of Sr^{85} absorption by lactose, further attention was given to this segment as a test site of lactose action. Solutions containing Sr^{85} and carrier calcium with or without lactose were injected directly into ligated ileal segments and the time relationship of accumulation of Sr^{85} by the femur was determined. The data in figure 2 show that a lactose effect was apparent at 30 minutes as judged by femur uptake of Sr^{85}. At this time interval, however, the difference between the Sr^{85} in the femurs of the lactose-injected and control rats was significant only at the probability levels of $0.05 < p < 0.10$. At one hour the influence of lactose was more pronounced and, at two hours, the differences were highly significant ($p < 0.01$). In this same experiment, the removal rate of Sr^{85} from the ileal segments was also measured and this constituted a direct estimate of net absorption. In the controls,

nearly all of the absorption of Sr^{85} took place during the first 30 minutes. In the presence of lactose, Sr^{85} absorption continued at a rapid rate to a maximum value at two hours. The lactose effect was highly significant ($p < 0.01$) at all time periods including the 30-minute interval. While absorption had virtually ceased at 30 minutes and two hours for the non-lactose and lactose-treated animals, respectively, the femurs continued to accumulate Sr^{85} in both groups over the 4-hour experimental period. This can be accounted for by re-distribution of Sr^{85} from the soft tissue to bone.

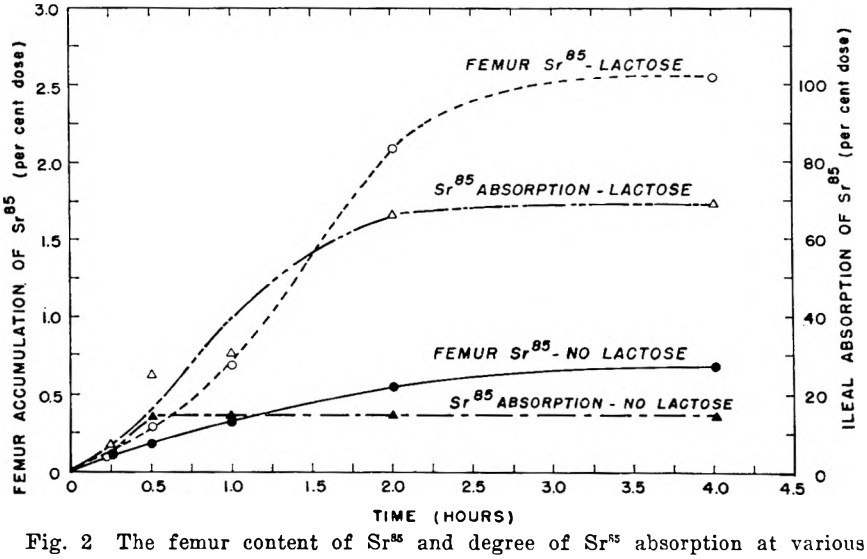

Fig. 2 The femur content of Sr^{85} and degree of Sr^{85} absorption at various time intervals after the ileal injection of a test solution with or without lactose.

In order to determine whether vitamin D potentiates or is essential for the lactose effect, rachitic rats were given vitamin D and lactose both separately and in combination. As shown in table 4, lactose alone and vitamin D alone each stimulated Sr^{85} absorption ($p < 0.05$). When administered in combination, the effect was no greater than when lactose was administered alone. Since the lactose effect was approximately the same with or without vitamin D in these deficient

rats, it is suggested that the presence of vitamin D is not essential for the action of lactose.

TABLE 4

The influence of lactose and vitamin D on the accumulation of orally administered Sr^{85} in the femurs of vitamin D-deficient rats [1]

TREATMENT	SR^{85} UPTAKE BY RAT FEMUR	PERCENT INCREASE IN SR^{85}
	% dose/femur	
$CaCl_2$ alone	1.5 ± 0.1	0
$CaCl_2$ + lactose	2.4 ± 0.1	60
$CaCl_2$ + vitamin D	2.0 ± 0.2	33
$CaCl_2$ + lactose + vitamin D	2.5 ± 0.1	67

[1] Values represent mean ± standard error of the mean; 7–9 animals per group; dose contained 5 mg $CaCl_2$ and 0.42 millimoles of lactose in 1 ml solution; 2000 I.U. vitamin D (Viosterol) given 48 hr. prior to experiment as indicated; experimental time was 24 hr.

DISCUSSION

The technique employed in the present studies could profitably be used for investigating further the mechanism by which lactose and similar substances promote calcium and strontium absorption from the gastrointestinal tract. The method, in essence, was to inject a solution containing Sr^{85} and the test substance directly into a ligated segment of the rat intestinal tract; in this study the ileum was used. At two hours, the rat is killed and the excised femur is counted without further processing in a well-type scintillation detector. Since Sr^{85} decays by way of 0.51 mev. gamma emission, this type of measurement is quite feasible; Sr^{85}, while not acting quantitatively identically to calcium, could be relied upon to give qualitatively valid results (table 1). The ileal segment was chosen because it was shown in the present study to respond maximally to lactose stimulation as compared with other intestinal segments. If desired, the amount of Sr^{85} that moves out of the ileal segment could be readily measured directly; however, the Sr^{85} content of the femur would indicate absorbed calcium that is actually available for bone salt formation. With the use of Sr^{85}, it is also

possible to detect bone accumulation of activity by external counting methods.

It has been recognized for some time (Bergeim, '26b) that calcium is well absorbed from the upper portions of the small intestine and less efficiently from the ileum; these observations are in agreement with the present studies. The observation that lactose has such a profound effect upon the ileal absorption of calcium is a new finding. Since the total effect of lactose in increasing calcium absorption is about a factor of two, and the relative enhanced absorption from gut segments was found to be 1.4 for the upper tract and 4.0 for the ileum, it may be reasoned that the main anatomical site of lactose action is the ileum. This would mean that the calcium that has escaped absorption in the upper portions of the gut is to a large extent that calcium acted upon by lactose. This is reminiscent of the suggestion that the ileum is the important site for the primary action of vitamin D in promoting the absorption of calcium (Harrison and Harrison, '51). Previous studies (Wasserman et al., '57; Lengemann et al., '57) have shown that vitamin D-deficient chicks and rabbits failed to respond to lactose (as a part of skim milk powder); this would indicate that the ileums of these species do not act in the same manner as those of the rat. It would be of interest to investigate further the metabolic or physiological differences in the ileums of the species that respond to lactose in comparison to those that do not.

The present results tend to eliminate the possibility that lactose acts by providing substrate for acid fermentation by bacteria. First of all, the time for the appearance of an increase in calcium absorption due to lactose seems too short for a significant amount of sugar fermentation to have occurred. In the ileal segment, a difference was apparent within 30 minutes. Also, since the animals had been raised on a lactose-free ration, it seems unlikely that they should possess an intestinal flora that readily attacks lactose. In addition, a subsequent experiment has shown that rats fed a diet containing 1% of sulfadiazine for one week responded to the

enhancing effect of lactose to about the same degree as animals fed the same diet without the added antibacterial agent.

It was of interest to observe that NaEDTA at certain concentrations completely suppressed the action of lactose. The maximum effect of NaEDTA was realized at levels that would completely complex all of the calcium and strontium ions into the tightly bound CaEDTA or SrEDTA chelate. NaEDTA *alone* at comparable levels given either orally or by intraperitoneal injection did not alter the pattern of Sr^{85} deposition in the femur. It would thus seem that lactose acts only on ionized or readily ionizable calcium or strontium. Solubilization is not a factor since the EDTA chelates are, in fact, soluble complexes. Although it may be fortuitous that Sr^{85} alone and Sr^{85} in the presence of NaEDTA are absorbed to about the same extent, this similarity in absorption may represent a component of the total absorption mechanism of alkaline earths that is dependent primarily on a diffusion-like mechanism.

No definitive statements in regard to the mechanism by which lactose acts on the calcium transport mechanism are warranted from the present observations. The extensive work of Fournier and his co-workers ('55) indicated that intestinal pH was not a major factor and suggested the idea that lactose, xylose, arabinose and galactose exerted their effects on ossification. The experimental approach of Fournier's group differed from the present studies in that under their conditions the sugars were ingested by the experimental animals over long periods of time and these sugars (termed "structural carbohydrates") may have improved calcification by providing a favorable stimulus or substrate for bone metabolism. In our studies, the conditions are such that lactose could hardly have elicited its response through an effect on bone. This is shown clearly in figure 2 where the ratio between femur Sr^{85} and absorbed Sr^{85} is not altered appreciably in the presence of lactose. Also, the rapidity of the lactose response and the manner by which singly admin-

istered bone-seeking radioisotopes enter the skeleton (largely by ion exchange and adsorption phenomena) in itself would discount any skeletal effect of lactose. Therefore, it seems that lactose has a direct action either on the gut wall or within the intestinal lumen.

Possible mechanisms to explain the action of lactose on calcium absorption range from purely physico-chemical concepts, for example, the binding capacity of lactose for calcium ions (Herrington, '34) to metabolic effects. These matters are still under study.

SUMMARY

1. The influence of lactose on Ca^{45} and Sr^{85} absorption was studied in the rat using a simple technique based on the appearance of the radionuclides in the femur after oral administration.

2. Lactose enhanced calcium absorption when the latter was given as either the chloride, gluconate, lactate, or acetate salt.

3. The chelating agent, sodium ethylenediaminetetraacetate (NaEDTA) had no effect on Sr^{85} absorption, but at high enough concentration was able to suppress completely the action of lactose.

4. Lactose increased calcium absorption in the absence or presence of vitamin D in the rachitic rat and did so as effectively as vitamin D.

5. In the intact animal the enhanced absorption of Ca^{45} and Sr^{85} due to lactose was noted within two hours after gavage. The increased gastric emptying time in the presence of lactose was not alone sufficient to account for the lactose action.

6. When solutions were injected into ligated segments of the gastrointestinal tract, it was observed that lactose was most effective in the ileum although an appreciable response was seen in the duodenum and jejunum.

7. The lactose effect was apparent within 30 minutes following injection into the ileum. Without lactose, all of the

absorption of Sr^{85} took place within 30 minutes, whereas, in the presence of lactose, absorption continued for two hours.

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INTERCONVERSIONS OF POLYUNSATURATED FATTY ACIDS BY THE LAYING HEN[1]

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INTRODUCTION

It has been shown by many workers that linoleic acid can be converted to arachidonic acid in the animal body and that some synthesis and interconversion of fatty acids occur in the body (Widmer and Holman, '50; Reiser, '50). Mead and coworkers ('53) proved that isotopic acetate can be demonstrated in the arachidonic acid molecules synthesized from linoleic acid.

The high fat content and rapid production of eggs make the laying hen a particularly suitable animal for the study of fat metabolism. As early as 1934, Cruickshank ('34) appreciably altered the unsaturated fatty acid content of egg yolk fats by feeding the hens linseed or hempseed oils. Reiser ('51) showed that addition of dienoic acid to an otherwise fat-free diet of the hen resulted in an increase in dienoic and pentaenoic acids and a possible increase in tetraenoic acid in the egg. According to that study the trienoic and hexaenoic acids do not increase. The addition of trienoic acid resulted in increases in all polyunsaturated acids having from two to 6 double bonds.

Fisher and Leveille ('57) reported that when hens were fed a ration containing 20% of safflower oil, the iodine number

[1] Supported, in part, by grants from the Robert A. Welch Foundation, Houston, Texas and the Marco Chemical Company, Fort Worth, Texas.

of the yolk fat increased from 74 to 103 and linoleic acid content from 8.9 to 18.4%. They did not report changes in higher unsaturated acids nor did they analyze the triglycerides and phospholipides separately.

Acting on the theory that unsaturated fatty acids lower blood cholesterol (and by inference that this, in turn, reduces the incidence or severity of atherosclerosis) Horlick and O'Neil ('58) have suggested that the level of unsaturated acids in the diet might best be increased by producing eggs and other dietary constituents with high levels of these fatty acids. They, therefore, fed laying hens a ration containing 10% of sunflower seed oil and found that after 10 days the linoleic acid of the egg fat rose from the stock feed level of 5.65 to 30.4% and arachidonic acid from 1.28 to 2.54%. There was some evidence that the ingestion of eggs high in the essential fatty acids reduced the rate of return of blood cholesterol to high control levels after being reduced by low-fat and soybean oil diets.

Reiser ('51) showed that the level of egg triglyceride linoleic and arachiodonic acids may be reduced to about 1 and 0.0% respectively, and the phospholipide acids to 2.7 and 2.2% respectively from hens fed a rigid fat-free diet for a year.

The present study is an attempt to determine the maximum levels to which the polyunsaturated acids in eggs may be raised by the inclusion of linoleic acid in the diet of the hen.

EXPERIMENTAL

Two White Leghorn hens in their 7th month of production, were placed in individual cages, supplied with running water, and fed a fresh commercial laying mash [2] daily. The temperature of the room was approximately 75°F. Feed consumption was between 0.20 and 0.25 pounds per hen per day. The composition of the stock diet as given by the manufacturer [2] was as follows: crude protein, not less than 16.0%;

[2] Ralston-Purina Layena Mash, Ralston-Purina Co., St. Louis, Mo.

crude fat, 3.2%;[3] crude fiber not more than 6.0%; nitrogen-free extract, not less than 50.0%.

The fatty acid composition of the stock ration fat was: saturated 61.5%; oleic 16.4%; linoleic 17.3%; linolenic 4.8%; arachidonic 0.0%. The iodine number of the fat was 66.0.

The fatty acid composition of the triglycerides of eggs from hens I and II are given in table 1 and for the corresponding phospholipides in table 2.

RESULTS AND DISCUSSION

It may be seen from tables 1 and 2 that the iodine number of the triglyceride reached a maximum of about 100 after 7 days on the 10% safflower oil ration and that this did not change when the level was raised to 20%. Simultaneously, the oleic acid content of the triglycerides decreased. This confirms the observations of previous workers that the unsaturated acids have been deposited mainly by substitution for oleic acid (Cruickshank, '34; Fisher and Leveille, '57).

On the stock diet the linoleic acid content of the triglyceride fatty acids of the egg yolk was found to be about 10%. This quickly rose to over 30% by the 5th egg laid on the 6th day after the hens began to ingest about 7.5% linoleic acid in their ration. It was not further increased by increasing the linoleic acid of the diet to 15%. It thus seems that there is a limit of 30% to which hens can incorporate dietary linoleic acid into yolk triglycerides, and that there is a homeostatic mechanism which controls the limits of polyunsaturated fatty acid deposition. The excess must be hydrogenated to saturated acids or preferentially metabolized.

The trienoic acid content of the egg yolk triglycerides remain nearly constant. This confirms the observation of Reiser ('51) that trienoic acids are not produced from dienoic acids in hens. A slight and transient increase with dietary linoleic acid at the 15% level is also apparent in the phospholipides. This increase is probably not linoleic acid but an intermediate in arachidonic acid synthesis, such as 6, 9, 12 octadecatrienoic.

[3] Determined by analysis.

TABLE 1

Composition of triglyceride fatty acids of egg yolks produced by hens ingesting diets containing 0.55%, 7.5% and 15% of linoleic acid

DAY NO.	EGG NO.		IODINE NO.		SATURATED		OLEIC		DIENOIC		TRIENOIC		TETRAENOIC		PENTAENOIC	
	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2
					%	%	%	%	%	%	%	%	%	%	%	%
					Stock diet — (0.55% linoleic acid)											
1	1	—	74.9	—	42	—	44	—	12.5	—	1.0	—	0.56	—	0.22	—
2	2	1	80.7	78.4	33	39	56	45	9.4	13.4	1.0	0.90	0.44	0.74	0.15	0.20
4	3	2	60.5	69.9	52	44	37	44	9.2	10.4	0.8	0.85	0.56	0.57	0.14	0.10
13	7	6	73.3	63.0	38	49	51	42	8.5	7.9	1.1	1.04	0.57	0.44	0.16	—
					Stock diet + 10% safflower oil — (7.5% linoleic acid)											
1	1	1	71.6	69.9	41	42	49	49	8.6	7.8	1.2	1.1	0.40	0.63	0.22	—
2	2	—	78.3	—	37	—	50	—	11.6	—	1.2	—	0.63	—	0.18	—
3	—	2	—	84.1	—	32	—	55	—	12.1	—	1.1	—	0.38	—	—
4	3	3	75.4	91.4	46	29	35	53	17.2	16.6	1.2	1.0	0.46	0.48	0.14	0.20
5	4	4	93.3	84.8	34	41	43	38	20.6	20.4	1.4	1.1	1.05	0.55	—	0.10
6	5	—	94.9	—	45	—	21	—	31.4	—	1.0	—	1.80	—	—	—
7	6	—	95.6	—	40	—	28	—	29.1	—	1.2	—	1.23	—	—	—
8	—	5	—	99.7	—	30	—	42	—	25.6	—	1.0	—	0.72	—	0.18
9	7	—	100.6	—	41	—	24	—	32.6	—	1.2	—	1.46	—	—	—
10	8	—	104.3	—	34	—	31	—	32.6	—	1.1	—	0.78	—	0.28	—
12	9	—	103.5	—	34	—	32	—	31.6	—	1.1	—	1.20	—	—	—
13	—	6	—	99.6	—	31	—	41	—	25.8	—	1.1	—	0.96	—	0.25
14	10	7	103.1	101.9	34	32	32	38	31.7	27.1	1.2	1.1	0.65	1.04	0.20	0.24
					Stock diet + 20% safflower oil — (15.0% linoleic acid)											
2	1	1	103.2	99.0	33	33	32	38	32.2	26.6	1.1	1.0	0.72	0.93	0.23	0.27
5	3	2	96.8	103.5	42	31	23	39	32.8	29.2	1.3	1.0	1.00	0.83	0.21	0.12
6	—	3	—	98.3	—	36	—	33	—	29.1	—	1.0	—	0.74	—	0.09
7	4	—	—	—	—	—	—	—	33.4	—	2.1	—	1.41	—	—	—
9	5	4	—	—	—	—	—	—	30.5	30.4	1.7	1.7	1.98	1.89	0.63	0.56
10	—	5	—	102.4	—	34	—	34	—	29.9	—	1.2	—	1.11	—	0.18
11	6	—	99.1	—	38	—	31	—	27.5	—	1.9	—	1.73	—	0.45	—
13	—	7	—	105.4	—	30	—	39	—	29.7	—	1.0	—	0.74	—	0.13
14	8	8	98.9	98.8	35	37	34	30	29.0	37.1	1.1	1.1	0.72	0.69	0.12	0.12

TABLE 2

Composition of phospholipide fatty acids of egg yolks produced by hens ingesting diets containing 0.55%, 7.5% and 15% of linoleic acid

DAY NO.	EGG NO.		DIENOIC		TRIENOIC		TETRAENOIC		PENTAENOIC		HEXAENOIC	
	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2	Hen 1	Hen 2
			%	%	%	%	%	%	%	%	%	%
			Stock diet — (0.55% linoleic acid)									
1	1	—	16.5	—	3.0	—	6.86	—	1.95	—	1.75	—
2	2	—	14.7	—	3.6	—	6.23	—	1.77	—	1.62	—
4	3	—	15.5	—	2.0	—	7.43	—	2.12	—	1.80	—
6	—	3	—	15.9	—	2.2	—	7.68	—	2.13	—	2.33
7	4	—	13.6	—	2.0	—	6.21	—	1.41	—	1.86	—
9	—	4	—	15.1	—	2.3	—	5.82	—	1.32	—	1.56
11	—	5	—	14.7	—	2.9	—	7.05	—	1.70	—	2.02
13	7	6	14.3	15.0	2.4	3.6	5.51	3.75	1.24	0.88	1.45	1.27
			Stock diet + 10% safflower oil — (7.5% linoleic acid)									
1	1	1	16.0	17.3	3.7	3.6	4.90	5.38	1.15	1.14	1.51	1.47
2	2	—	16.7	—	2.5	—	4.91	—	1.09	—	1.35	—
3	—	2	—	16.7	—	3.7	—	5.70	—	1.23	—	1.61
4	3	3	19.0	18.9	3.3	4.1	4.90	6.22	1.11	1.34	1.22	1.58
5	4	4	24.3	21.5	2.9	4.0	6.65	7.06	1.52	1.37	1.66	1.52
6	5	—	23.4	—	3.7	—	6.72	—	1.46	—	1.36	—
7	6	—	25.2	—	3.3	—	6.43	—	1.46	—	1.48	—
8	—	5	—	24.2	—	3.5	—	6.22	—	1.49	—	1.22
9	7	—	26.1	—	3.6	—	6.27	—	1.43	—	1.61	—
10	8	—	28.0	—	3.2	—	6.08	—	1.44	—	1.29	—
12	9	—	29.1	—	3.6	—	6.73	—	1.28	—	1.21	—
13	—	6	—	23.7	—	3.0	—	6.74	—	1.58	—	1.34
14	—	7	—	24.2	—	1.6	—	8.44	—	2.24	—	1.71
			Stock diet + 20% safflower oil — (15.0% linoleic acid)									
2	1	1	29.5	22.3	2.8	3.5	5.51	6.59	1.46	1.75	1.12	1.79
4	2	—	28.7	—	3.1	—	6.71	—	1.70	—	1.34	—
5	3	2	31.2	27.2	1.2	3.4	8.46	7.61	2.70	1.83	1.77	1.57
6	—	3	—	30.1	—	1.7	—	8.27	—	3.07	—	—
10	—	5	—	30.2	—	1.6	—	8.19	—	2.67	—	—
13	7	7	27.3	25.8	1.8	2.2	9.32	10.97	3.20	3.89	—	—
14	8	8	24.0	26.7	1.5	2.1	8.75	8.27	3.42	2.44	—	—

The triglyceride acids in eggs from hens fed the stock diet contained about 0.5% of arachidonic acid. With the use of the 10% safflower oil diet (7.5% linoleic acid) the arachidonic acid level gradually increased to a maximum approaching 2% and returned to the basal level while the hens remained on the high linoleic acid regimen. The increase in the level of safflower oil in the diet to 20% (15% linoleic) stimulated a second rise toward 2% followed by a return to the basal level.

These results confirm the conclusions of previous workers that hens can convert dietary linoleic acid to arachidonic acid. However, the degree of conversion is limited and transient and does not seem to be dependent on the amount of linoleic acid fed, at least at the higher levels.

The pentaenoic acid content of the egg yolk triglycerides appears not to be influenced by the levels of dietary linoleic acid used in this study.

The linoleic acid content of the phospholipide of eggs produced by hens ingesting the stock diet was about 15% (table 2). With 7.5% linoleic acid in the diet, it gradually increased, approaching an apparent upper limit of 30%. Doubling the dietary linoleic acid to 15% did not raise it further. Conversely, there is evidence that the level began to drop after about two weeks on the 15% linoleic acid diet.

The trienoic acid showed a similar behavior. With the 7.5% linoleic acid diet it increased a little over the stock diet, but it did not maintain this increase and returned to its original level.

It may be noticed that phospholipide tetraenoic acid did not decrease on the low dietary linoleic acid until the 13th egg. This resistance to depletion is in confirmation of previous work from this laboratory (Reiser, '51). The response to the addition of 7.5% linoleic acid in the diet was reasonably rapid but quickly leveled off, with a second increase to a maximum of 10% at the 15% dietary linoleic acid intake.

The phospholipide pentaenoic and hexaenoic acid content showed no change on any of these levels of dietary linoleic acid.

CONCLUSIONS

1. The dienoic acid level of egg yolk triglycerides and phospholipides approaches an upper limit of 30% by the inclusion of not over 7.5% of linoleic acid in the diet.

2. The trienoic acid level of triglycerides is not influenced by levels of dietary linoleic acid above 0.55%, except a slight transient increase at the 15% level in the diet.

3. The trienoic acid level of phospholipides increases from a basal value of 1.0% toward a maximum of 4% on a dietary level of 7.5% linoleic acid, but does not maintain the increase even at the 15% level in the diet.

4. The tetraenoic acid level of triglycerides, upon the ingestion of diets containing 7.5 or 15% of linoleic acid initially increases from a basal value of about 0.5% toward an upper limit of 2%, but, after several eggs, spontaneously returns to the basal level.

5. The tetraenoic acid of phospholipides changes slowly with dietary linoleic acid but increases toward an upper limit of 10%.

6. The pentaenoic acid of the triglycerides and phospholipides, and the hexaenoic acid of phospholipides are not influenced by dietary linoleic acid levels above 0.55%.

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ENDEMIC GOITRE OF THE ISLAND OF KRK STUDIED WITH I^{131}

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In a previous study from this laboratory (Maver and Horvat, '58) concerning the examination of goitre on the islands of the Adriatic, a relatively high incidence of goitre on the island of Krk was described. In general, the northern parts of the island around the villages of Polje and Risika are more heavily afflicted with goitre than the southern parts (the town of Krk), whereas the south-western part of the island (Milohnići) is almost goitre-free. The data about the incidence of goitre among the population showed that goitre was present in about 46% of the population of Risika, in 40% at Polje, while the incidence of goitre at Milohnići did not exceed 3%. According to the high incidence of goitre in the villages of Risika and Polje both these areas may be considered as areas of endemic goitre.

An incidence of goitre on islands being rather unusual, a study — using radioactive iodine — was undertaken with a view to following the thyroidal uptake and urine excretion of I^{131} as well as the excretion of stable iodine in subjects from various parts of the island.

[1] The authors are indebted to the Council of Public Health of the People's Republic of Croatia in Zagreb for the research grant, and to Dr. Gudac of the Health Center at Krk for the support in the organization of this field trial.

METHODS

The subjects were chosen among the euthyroid inhabitants of Risika, Polje, Milohnići, and the town of Krk. They were mostly women with goitre of grade I, according to the classification of the Goitre Commission of W.H.O. ('53) and a few cases of grade II goitre. In most cases goitre belonged to the type of *struma diffusa parenchymatosa* with a few cases of *struma nodosa.* The subjects passed a physical check-up before the application of I^{131}, but there was no opportunity to determine the basal metabolic rate. The protein-bound iodine was not determined during this study inasmuch as a preliminary survey had shown the values to be within normal range.

As controls nongoitrous subjects from the town of Krk and normal subjects from Zagreb were chosen. A certain number of subjects from both the experimental and the control group were hospitalized and 48-hour samples of urine were collected. Other subjects were transported to the clinic by car every day.

The thyroidal uptake of I^{131} was followed during 3, 6, 12, 24 and 48 hours after the oral application of a radioactive dose, by means of a Geiger Mueller tube. The radioactivity of urine was counted in a Marinelli beaker in adequate portions of 48-hour urine. The determination of stable iodine in urine was performed by the Barker ('48) method, as modified in Stanbury's laboratory.

RESULTS

The 3, 6, 24 and 48-hour thyroidal I^{131} uptake is presented in figure 1. The highest uptake of I^{131} is shown by the goitrous subjects from the village of Risika who reached their maximum of 57.1% in 24 hours. After 24 hours a slight decline took place which at the 48-hour limit showed a value of 54.6%. The curve of the subjects from Polje followed the described curve for the subjects from Risika very closely during the first 24 hours, but there it was not possible to

follow these subjects through the whole 48-hour interval. The goitrous subjects from the town of Krk showed a less rapid I^{131} uptake with a maximum of 43.9% after 24 hours. The nongoitrous subjects from the town of Krk showed the same type of curve as the previous group, but on a lower level. The maximum uptake after 24 hours was 40.5%. The lowest uptake among the subjects on the island of Krk was shown by the group from Milohnići. Their 24-hour uptake did not exceed 36% of the administered dose.

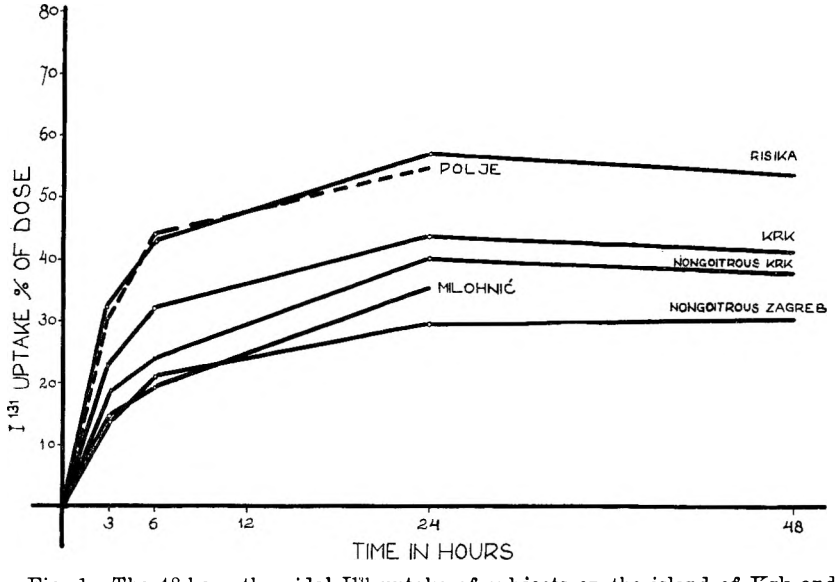

Fig. 1 The 48-hour thyroidal I^{131} uptake of subjects on the island of Krk and of a control group in Zagreb.

Normal persons from Zagreb had a thyroidal I^{131} uptake of 32.9% after 24 hours and their curve, unlike that of the subjects on the island of Krk, continued to rise slightly during the next 24 hours to reach its maximum after 48 hours, at the level of 34%.

The urinary excretion of radioactive and stable iodine of the hospitalized group from Risika and of the control group from Zagreb are presented in table 1. The excretion of both

radioactive and stable iodine was lower among the subjects from Krk than in the normal group from Zagreb.

TABLE 1

The urinary excretion of I^{131} and I^{127} by goitrous subjects from the island of Krk and of controls from Zagreb

	48-HOUR URINARY I^{131} EXCRETION		24-HOUR URINARY I^{127} EXCRETION	
	Mean ± S.D.[1]	Range	Mean ± S.D.	Range
	%	%	μg	μg
Goitrous subjects from Polje and Risika	38.7 ± 14.0	20.4–65.0	26.7 ± 11.3	12.9–44.6
Nongoitrous subjects from Zagreb	45.2 ± 17.3	18.0–75.0	44.3 ± 19.3	24.2–78.6

[1] Standard deviation.

DISCUSSION

A study of the thyrodial I^{131} uptake in areas of endemic goitre on the island of Krk showed an increased avidity of the thyroid for iodine. The curve of radioiodine uptake reached its peak after 24 hours showing that in this interval 57.1% of the radioactive dose administered to the subjects from Risika and 55.4% of the dose given to the subjects from Polje were removed from the blood by the thyroid. In the first 6 hours 80% of the total uptake of the I^{131} was already trapped in the gland. The radioactive iodine uptake of normal subjects from Zagreb showed a different type of curve which reached its maximum after 48 hours with a mean of 34.8% of the initial dose. These values of I^{131} uptake are almost identical with the results of Stanbury et al. from the area of endemic goitre in Mendoza as well as for normal subjects from Boston (Stanbury et al., '54).

The curves of the I^{131} uptake presented in figure 1 fairly reflect the epidemiological picture of the incidence of goitre on the island. The highest was the uptake in both areas of endemic goitre (Risika and Polje), while the southern part

around the town of Krk which is less goitrous showed an uptake of 45% for goitrous and 40% for nongoitrous subjects. The subjects from Milohnići, practically a goitre-free area, showed an I^{131} uptake with a mean of 36%. The curves in figure 1 show that goitre occurred when the 24-hour I^{131} uptake by the thyroid had exceeded 41%. There was no goitre in the areas where the 24-hour I^{131} uptake was below this level. Werner ('57) came to a similar conclusion in a study on more than 2,000 subjects who showed that a 24-hour I^{131} uptake of 55% was always diagnostic of hyperthyroidism or hyperplasia, provided there were no complicating factors, since values below 40% uptake practically excluded such disturbances. This range cannot be used as normal since it depends on the type of counter employed.

In comparing the values for I^{131} uptake between normal subjects from Zagreb and the values for the island of Krk, it is evident that among the subjects from all examined areas of the island the I^{131} uptake was higher than in Zagreb controls. Thus the whole examined area of the island might be considered as an area of disturbed iodine metabolism, resulting in the appearance of endemic goitre in the northern and of sporadic goitre in the southern parts, whereas the southwestern parts remained almost goitre-free.

The iodine supply on the island was lower than in the nonendemic area of Zagreb, according to urinary excretion of I^{131} (table 1). The 24-hour excretion of I^{127} among the subjects from Risika and Polje was 26.7 μg in contrast to 44.5 μg among the subjects in Zagreb. If an euthyroid person daily excretes iodine in the amount of the daily intake and 90% of iodine excretion is eliminated from the body through urine, the daily intake of iodine in both areas can be calculated. Thus, 30 μg of iodine would represent the daily iodine supply of a person at Risika or Polje in comparison with about 50 μg in Zagreb. A supply of 30 μg of iodine daily is very low, and even 50 μg still represents a low intake, for according to Stanbury ('58) endemic

goitre will appear if the daily supply of iodine falls below 70 μg.

In spite of the existing difference in the incidence of goitre between the various parts of the island, there was, however, no evidence of a difference in the iodine supply in water or food between the examined areas of the island of Krk (Horvat and Maver, '58). Thus it does not seem unlikely that other factors might play a role in the development of goitre on this island. In an attempt to examine the presence of eventual goitrogenic factors, 400 mg of potassium perchlorate were given to a group of 12 subjects from Polje and Risika 60 minutes after the I^{131} dose. In 5 subjects there was a decline in the radio iodine uptake curve 20 and 40 minutes after the administration of perchlorate, indicating that in those subjects the trapped iodine was not bound organically at that time.

SUMMARY

The 3, 6, 24 and 48-hour thyroidal I^{131} uptake and the urinary excretion of I^{127} and I^{131} were studied among subjects in the goitrous areas of the island of Krk. The results showed an avidity of the thyroid gland for radioactive iodine, the uptake ranging between 57% of the administered dose for more severe and 45% for less severe goitrous areas. Subjects from nongoitrous areas of the same island as well as controls from the nongoitrous area of Zagreb showed a thyroidal I^{131} uptake of less than 40%.

The urinary I^{127} and I^{131} excretions were lower among goitrous subjects from the island than among controls in Zagreb.

From these data it can be concluded that the incidence of goitre on the island of Krk is due to lack of iodine. However, according to an earlier study on the island of Krk no difference was found in the iodine supply between various areas. It could be assumed that some other factors impair the utilization of iodine in the goitrous areas, thus creating a relative deficiency.

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STUDIES ON B VITAMIN INTERRELATIONSHIPS IN GROWING RATS

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Although the existence of interrelationships between B vitamins has been recognized for many years, there is little direct information on whether the dietary level of one B vitamin affects the growth of animals receiving inadequate amounts of a second B vitamin. In experimentally produced deficiency disease, several investigators (György, '34; Chick et al., '35; Harris, '35) found that rats deficient in both riboflavin and pyridoxine did not exhibit the florid dermatitis characteristic of pyridoxine deficiency, unless adequate riboflavin was added to the diet. Lepkovsky et al. ('36) also showed that symptoms of acute pyridoxine deficiency did not appear in the rat unless adequate pantothenic acid was present in the diet. In early clinical studies with the B vitamins, it was observed that treatment of multiple deficiencies with a single vitamin sometimes resulted in the appearance of symptoms characteristic of deficiency of other vitamins (Sydenstricker, '41). Unna and Clark ('42) found no evidence that administration of large amounts of individual B vitamins to rats deficient in other B vitamins aggravated the symptoms of the specific deficiency involved. In studies on the interaction of various B vitamins in growing rats, Scott and Griffith ('58) reported a synergistic relationship between pantothenate and pyridoxine, in which the animals deficient in both vitamins grew much more slowly than those deficient in either one.

The present report deals with growth studies, in which male weanling rats received diets containing low, adequate and high dietary levels of each of several B vitamins, in the presence of low, adequate and high dietary levels of a second B vitamin. The effects of administration of high levels of individual B vitamins to rats deficient in three other B vitamins were also studied.

EXPERIMENTAL

The basal diet was similar to that used by Sarett and Snipper ('54) and contained 18% vitamin-free casein, 10% corn oil, 64% sucrose, 4% salts (Jones and Foster ('42) with NaF added), 3.65% fiber and the following vitamins per 100 gm: thiamine hydrochloride, 0.4 mg; riboflavin, 0.5 mg; niacinamide, 5.0 mg; pyridoxine hydrochloride, 0.25 mg; calcium pantothenate, 2.0 mg; choline bitartrate, 200.0 mg; inositol, 100.0 mg; *p*-aminobenzoic acid, 10.0 mg; folic acid, 0.20 mg; biotin, 0.02 mg; vitamin B_{12}, 0.01 mg; menadione, 0.2 mg; ascorbic acid, 20.0 mg; α-tocopherol, 5.0 mg; and oleum percomorphum, 0.015 ml. Modifications were made in the levels of those vitamins studied as described below.

In experiments 1 to 4, the interrelationships of low, adequate and high levels of various pairs of B vitamins were tested in growing rats. For example, in experiment 1, low, adequate and high levels of thiamine were each tested in the presence of low, adequate and high levels of pyridoxine. The vitamins tested, and the levels per 100 gm of diet designated as adequate in the 4 experiments, were as follows: experiment 1 — thiamine hydrochloride, 0.2 mg, pyridoxine hydrochloride, 0.2 mg; experiment 2 — thiamine hydrochloride 0.2 mg, calcium pantothenate, 2.0 mg; experiment 3 — thiamine hydrochloride, 0.2 mg, riboflavin, 0.4 mg; experiment 4 — riboflavin, 0.4 mg, calcium pantothenate, 2.0 mg. The low levels, chosen to produce marked deficiencies of the vitamins tested, were one-twentieth of the adequate levels, whereas the high or excess levels used were 50 times the adequate levels.

In experiment 5, the effects of administration of high levels of an individual B vitamin (thiamine, riboflavin, pyridoxine or pantothenate) to rats receiving diets low in the other three B vitamins, were tested. The levels of the 4 B vitamins chosen as adequate for this experiment were slightly different, namely, thiamine hydrochloride, 0.4 mg; riboflavin, 0.5 mg; pyridoxine hydrochloride, 0.25 mg; calcium pantothenate, 2.0 mg per 100 gm of diet. The low levels of the vitamins were one-fifth the adequate levels and the high levels were 50 times the adequate levels. In addition, ascorbic acid was not included in the vitamin mixture used in this experiment.

In each of the 5 experiments reported herein, equivalent groups of 10 male weanling rats (McCollum-Wisconsin strain) were selected on the basis of litter origin and body weight. The animals were from 19 to 21 days of age at the beginning of the experiments and weighed approximately 50 gm. The rats were housed in individual screen-bottom cages in an air-conditioned room maintained at 74 to 76°F. and were given the experimental diets and tap water ad libitum. Records were kept of the amounts of food and water consumed by each rat and the animals were weighed at weekly intervals during the experiments. In experiments 1 to 4, the severely deficient groups of animals were sacrificed after 4 weeks, whereas the others were continued on experiment for 7 or 8 weeks. In experiment 5, the deficiencies were less severe, permitting the experiment to be continued for 12 weeks.

At the end of each experiment, the animals were fasted for 24 hours, sacrificed by intraperitoneal injection of Nembutal [1] solution and the livers, kidneys and adrenal glands were removed and weighed. The only data on organ weights which show differences which warrant inclusion in this report are those on the adrenal glands of the animals in experiment 5.

RESULTS

Data on weight gains and food efficiency values obtained in experiments 1 to 4 are summarized in table 1. Although some

[1] Abbott.

TABLE 1

Data on effect of high levels of various B vitamins on weight gain and food efficiency of male weanling rats fed diets deficient in other B vitamins for 4 weeks

	DIET NO. AND DESCRIPTION	NO. OF SURVIVORS [1]	WEIGHT GAIN	FOOD EFFICIENCY
			gm	*gm gain/100 gm food*
	Experiment 1			
11.	Adequate control diet	9	120 ± 23 [2]	37.3 ± 3.3 [2]
12.	Low thiamine	9	6 ± 5	3.3 ± 2.5
13.	Low pyridoxine	9	46 ± 11	21.6 ± 4.5
14.	Low thiamine, pyridoxine	6	3 ± 1	1.9 ± 0.6
15.	Low thiamine, high pyridoxine	8	12 ± 7	6.7 ± 3.9
16.	Low pyridoxine, high thiamine	10	45 ± 6	21.4 ± 2.9
	Experiment 2			
21.	Adequate control diet	8	130 ± 11	41.5 ± 1.0
22.	Low thiamine	9	13 ± 8	9.1 ± 5.7
23.	Low pantothenate	9	83 ± 16	32.5 ± 3.1
24.	Low thiamine, pantothenate	9	12 ± 5	8.7 ± 3.8
25.	Low thiamine, high pantothenate	9	8 ± 6	5.5 ± 4.3
26.	Low pantothenate, high thiamine	9	70 ± 12	30.2 ± 3.1
	Experiment 3			
31.	Adequate control diet	10	119 ± 16	38.7 ± 3.3
32.	Low thiamine	8	7 ± 9	4.0 ± 5.2
33.	Low riboflavin	9	26 ± 9	16.3 ± 4.6
34.	Low thiamine, riboflavin	10	0 ± 4	0 ± 3.1
35.	Low thiamine, high riboflavin	10	12 ± 10	7.1 ± 6.1
36.	Low riboflavin, high thiamine	10	26 ± 6	15.4 ± 3.0
	Experiment 4			
41.	Adequate control diet	10	134 ± 10	40.4 ± 2.8
42.	Low riboflavin	10	30 ± 8	16.9 ± 3.2
43.	Low pantothenate	8	80 ± 11	30.2 ± 2.6
44.	Low riboflavin, pantothenate	10	34 ± 9	18.2 ± 4.3
45.	Low riboflavin, high pantothenate	10	38 ± 12	18.6 ± 3.7
46.	Low pantothenate, high riboflavin	10	82 ± 12	30.1 ± 2.9

[1] Each group contained 10 animals initially.
[2] Standard deviation.

of the groups were continued on experiment for 7 or 8 weeks, only 4-week data are presented in this table, in order to compare the deficient groups with the others. Curves of weight gain for the 4 experiments are shown in figure 1. Since addi-

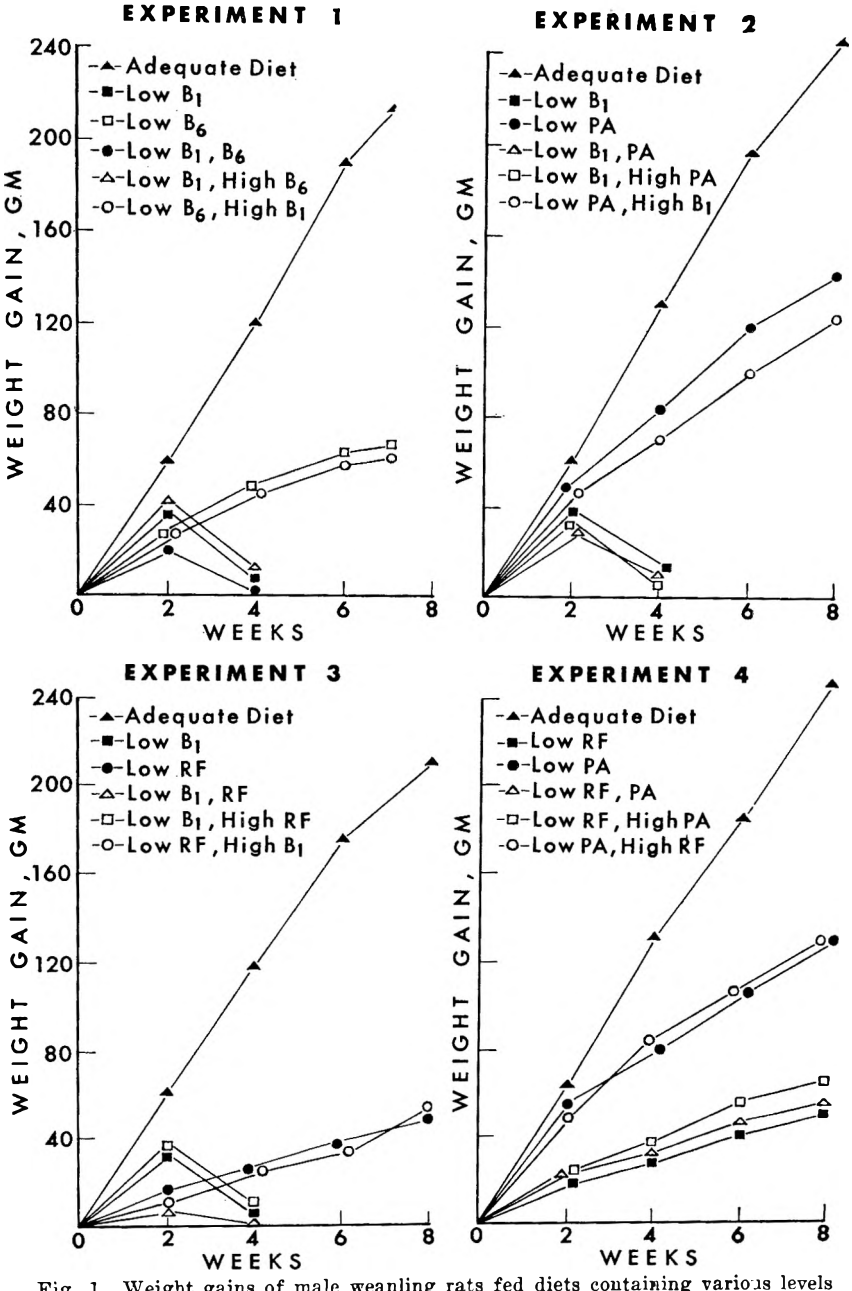

Fig. 1 Weight gains of male weanling rats fed diets containing various levels of thiamine (B_1), riboflavin (RF), pyridoxine (B_6) and pantothenate (PA).

tion to the adequate control diet of high levels of either or both of the vitamins tested did not significantly influence weight gain, the data for these groups are not reported herein.

In the first three experiments, the animals which received otherwise adequate diets containing the low level of thiamine (diets 12, 22 and 32) gained only 6, 13 and 7 gm, respectively; whereas, those given the corresponding adequate control diet (diets 11, 21 and 31) gained 120, 130 and 119 gm, respectively. Thiamine deficiency markedly reduced the efficiency of food utilization. The animals which received diets deficient in a second vitamin in addition to thiamine (pyridoxine, diet 14; pantothenate, diet 24; riboflavin, diet 34) grew at comparable rates to those given the corresponding diets deficient only in thiamine, indicating that thiamine deficiency was the primary factor limiting growth. The lack of influence of concomitant pantothenate deficiency on the severity of thiamine deficiency observed in the present studies is in agreement with the findings of Scott and Griffith ('58), but does not agree with the conclusions of Beznák and van Alphen ('55). The validity of the findings of the latter workers is questionable, since, in their studies, the rats on the pantothenate "deficient" diet grew as well as those on the control diet.

The addition of high levels of pyridoxine (diet 15), pantothenate (diet 25), or riboflavin (diet 35) had no significant effect on the weight gain or food efficiency of animals receiving a low level of thiamine. It is possible that with less severe deficiency, continuation of the experiments for a longer period of time might have disclosed some effect of vitamin excesses.

The growth depressing effects of low levels of pyridoxine (diet 13), pantothenate (diets 23 and 43) or riboflavin (diets 33 and 42) were apparent after 4 weeks on experiment (table 1) and were even more marked after 7 or 8 weeks (fig. 1).

The addition of high levels of thiamine to the diet had no significant influence on the weight gain in pyridoxine deficiency (diet 16 vs. diet 13), pantothenate deficiency (diet 26 vs. diet 23), or riboflavin deficiency (diet 36 vs. diet 33).

TABLE 2

Data on average weight gains, food efficiencies and adrenal weights of male weanling rats fed diets containing combinations of various levels of thiamine (B_1) riboflavin (RF), pyridoxine (B_6) and pantothenate (PA) for 12 weeks

Experiment 5

	DIET NO. AND DESCRIPTION	NO. OF SURVIVORS [1]	WEIGHT GAIN	FOOD EFFICIENCY	ADRENAL WEIGHT	
			gm	*gm gain/100 gm food*	*mg*	*mg/100 gm body wt.*
51.	Adequate control diet	9	299 ± 25 [2]	25.9 ± 1.7 [2]	49.2	14.6 ± 2.4 [2]
52.	Low B_1, RF, B_6, PA	10	62 ± 30	12.0 ± 4.7	29.5	28.8 ± 3.9
53.	Adequate B_1, low RF, B_6, PA	10	143 ± 38	19.7 ± 3.2	36.8	20.4 ± 3.3
54.	High B_1 low RF, B_6, PA	10	141 ± 29	20.1 ± 3.1	33.9	19.3 ± 2.8
55.	Adequate RF, low B_1, B_6, PA	9	101 ± 40	15.9 ± 3.9	33.2	24.6 ± 4.8
56.	High RF, low B_1, B_6, PA	4	60 ± 25	11.7 ± 3.5	28.2	25.9 ± 4.1
57.	Adequate B_6, low B_1, RF, PA	10	103 ± 43	16.5 ± 3.7	30.3	22.1 ± 4.5
58.	High B_6, low B_1, RF, PA	10	86 ± 35	14.3 ± 4.4	28.3	22.6 ± 4.3
59.	Adequate PA, low B_1, RF, B_6	10	80 ± 43	13.9 ± 6.0	30.9	26.0 ± 3.9
60.	High PA, low B_1, RF, B_6	10	94 ± 44	15.1 ± 5.9	30.6	24.1 ± 6.3
61.	High B_1, RF, B_6, PA	9	290 ± 26	24.7 ± 1.8	46.6	14.4 ± 1.9

[1] Each group contained 10 animals initially.

[2] Standard deviation.

Likewise, the severity of riboflavin deficiency was not influenced by the addition of high levels of pantothenate to the diet (diet 45 vs. diet 42), nor were the effects of pantothenate deficiency influenced by high levels of riboflavin (diet 46 vs. diet 43).

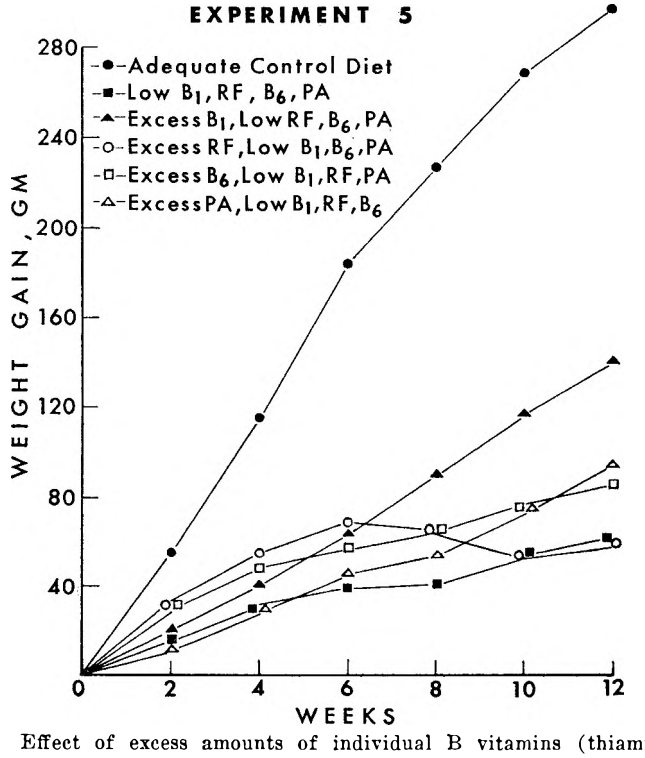

Fig. 2 Effect of excess amounts of individual B vitamins (thiamine (B_1), riboflavin (RF), pyridoxine (B_6) or pantothenic acid (PA)) on weight gain of male weanling rats fed diets deficient in three other B vitamins.

The results of experiment 5 are summarized in table 2. Curves of weight gain of the animals fed the control diet, low levels of all 4 vitamins tested and high levels of each of the 4 vitamins in the presence of low levels of the other three vitamins, are shown in figure 2. The animals fed the adequate control diet (diet 51) gained 299 gm during the 12-week experimental period, with an average food efficiency of 25.9 gm gain per 100 gm food intake. The addition to the adequate

control diet of high levels of thiamine, riboflavin, pyridoxine and pantothenate (diet 61) had no significant influence on weight gain or food efficiency. The diet which contained low levels of all 4 vitamins tested (diet 52) gave markedly reduced weight gain and food efficiency. The addition of a high level of thiamine to the diet deficient in the other three B vitamins studied (diet 54) increased weight gain the same as did an adequate level of thiamine (diet 53). The effects of high levels of pyridoxine (diet 58) or pantothenate (diet 60) were also similar to those of adequate levels (diets 57 and 59, respectively). However, the animals fed diet 56, which contained a high level of riboflavin and low levels of thiamine, pyridoxine and pantothenate, began to lose weight after 6 weeks on experiment (fig. 2). By 12 weeks (table 2), only 4 of the 10 animals started on this diet survived and their average weight gain (60 gm) and food efficiency (11.7) were significantly less ($P < 0.01$ by "t" test, Snedecor, '55) than the corresponding findings on diet 55, which contained an adequate level of riboflavin, and low levels of the other three vitamins tested.

The adrenals of the animals fed adequate or high levels of all 4 vitamins (diets 51 and 61, table 2) were normal in weight for rats of our strain fed good quality diets. The animals which received low levels of all 4 vitamins (diet 52) had significantly ($P < 0.01$) heavier adrenal glands, per unit of body weight, than those fed adequate or high levels of these vitamins. The adrenals of the animals fed low levels of only three of the 4 vitamins were intermediate in weight. Of the 4 vitamins tested, addition of thiamine produced the greatest decrease in relative adrenal weights, suggesting that thiamine deficiency was the primary limiting factor in the combinations studied.

Individual vitamin relationship studies, similar to those carried out in experiments 1 to 4, have also been conducted in this laboratory with vitamin B_{12} and folic acid, and vitamin B_{12} and pantothenic acid. The results showed that high levels of vitamin B_{12} had no effect on the weight gain or food effi-

ciency of rats deficient in folic acid or pantothenic acid. In another experiment, prompted by the studies of Dinning et al. ('54) on the relationships of pyridoxine and vitamin E, high levels of vitamin E had no effect on weight gain or liver cysteine desulfhydrase activity in animals receiving a low level of pyridoxine.

DISCUSSION

The results of the present studies confirm and extend the observations of Unna and Clark ('42), that administration of a high level of various individual B vitamins does not significantly influence the weight gains of animals deficient in other individual B vitamins. No evident sparing effects of high levels of one B vitamin on the requirement for another B vitamin were observed, nor did excess dietary supplementation with one vitamin increase the severity of deficiency of a second. The results of experiment 5 suggest that some multiple vitamin deficiencies may be adversely affected by high levels of a single vitamin. These results bring to mind the early clinical observations of Spies et al. ('39) and Sydenstricker ('41). Spies et al. found that pellagrins treated with nicotinic acid alone often acquired symptoms of beriberi, ariboflavinosis, or both, after the manifestations of pellagra had been relieved. The findings of Spies et al. may be related to the observations made in experiment 5, or to removal of one deficiency, permitting the development of symptoms of deficiency of the next most limiting vitamin.

The diets used in experiments 1 to 4 contained a low level (0.02%) of ascorbic acid, whereas no ascorbic acid was included in the diets in experiment 5. Since ascorbic acid, at levels of 2 to 5% of the diet, delays the appearance of symptoms of deficiency of certain B vitamins (Daft and Schwarz, '52; Barboriak and Krehl, '57), the findings in experiments 1 to 4 may have been slightly affected by the ascorbic acid in the diet. However, this appears highly unlikely, since the level used in the present studies was much less than that shown by other workers to influence B vitamin require-

ments. Experiments to determine whether small amounts of ascorbic acid have any effect on B vitamin interrelationships may, however, be warranted.

SUMMARY

B vitamin interrelationships were studied in growing animals by giving male weanling rats otherwise adequate diets containing low, adequate or high levels of one B vitamin, in combination with low, adequate or high levels of a second B vitamin. In these experiments, the low levels of vitamins tested were one-twentieth those chosen as adequate, whereas the high levels were 50 times the adequate levels. Deficiency of thiamine, riboflavin, pyridoxine or pantothenate significantly reduced weight gain and food efficiency. The animals deficient in both thiamine and in a second B vitamin (riboflavin, pyridoxine or pantothenate), grew at rates comparable to those deficient in thiamine alone. High levels of thiamine had no influence on the weight gain and food efficiency of the rats fed diets deficient in pyridoxine, pantothenate or riboflavin. Similarly, no effects of high pyridoxine, pantothenate or riboflavin levels on the severity of thiamine deficiency were observed. In addition, no evidence was found of any interrelationship between high and low levels of pantothenate and riboflavin.

A study was also made of the effects of administration of an excess level of thiamine, riboflavin, pyridoxine or pantothenate to animals deficient in the other three B vitamins. No adverse effects of high levels of thiamine, pyridoxine or pantothenate were observed. However, high dietary riboflavin significantly depressed weight gain and food efficiency, and increased the mortality of rats fed diets deficient in thiamine, pyridoxine and pantothenate.

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THE EFFECT OF A LOW ENVIRONMENTAL TEMPERATURE ON THE WEIGHT AND FOOD CONSUMPTION OF RIBOFLAVIN-DEFICIENT RATS [1,2]

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INTRODUCTION

The requirements for riboflavin and other B vitamins are commonly thought to be proportional to food intake. However, recent investigations (Mitchell et al., '50; Ershoff, '52) have cast some doubt on the validity of this concept when applied to animals subjected to a low environmental temperature. Riboflavin is known to be involved in the production of the adrenocortical hormones (Forker and Morgan, '54) which are concerned with the physiological response to cold (Tyslowitz and Astwood, '42). As part of the flavin coenzymes, it plays a role in the catabolism of fat, the importance of which may be enhanced in the cold (Chenier, '54). For these reasons, the requirement of rats for riboflavin might be dependent on factors other than food intake during periods of lowered environmental temperatures. However, Bessey et al. ('58) found that there was no increase in riboflavin destruction by riboflavin-deficient rats when metabolism was increased by cold exposure. In the same paper, these authors cite the work of Fontaine and Raffy ('42),

[1] Portions of this paper were presented before the Ninth Alaska Science Conference, Alaska Division AAAS, College, Alaska, September, 1958.

[2] The views expressed are those of the authors and do not necessarily represent Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association.

whose rats exposed to cold had a 20% higher concentration of riboflavin in the liver.

If food intake is the most critical determinant of riboflavin needs, animals subsisting in the cold, when allowed to fill their cold-induced energy requirements, should be as well off as their controls in the warm when eating the same levels of riboflavin per unit of food. The experiment presented in this report was planned to test this proposition.

EXPERIMENTAL

Fifty male Sprague-Dawley rats, ranging in weight from 175 to 250 gm were divided into two groups of 25 each. One group was placed in individual cages in a cold room held at $5 \pm 2°C$; the other group remained in an animal room at 25°C. Wire-bottom cages were used, and the rats were randomized as to position in the cage rack. At this time, all rats were placed on a riboflavin-deficient basal diet [3] until growth ceased or fluctuated around a central point. The riboflavin-deficient diet had the following composition: vitamin test casein, 18%; sucrose, 68%; vegetable oil, 10%; U.S.P. Salt Mixture no. 2, 4%. The vitamin mixture supplied 2000 units of vitamin A, 222 units of vitamin D, 11.1 mg of α-tocopherol, 100 mg of ascorbic acid, 11.1 mg of inositol, 166.5 mg of choline chloride, 5 mg of menadione, 11.1 mg of *p*-aminobenzoic acid, 10 mg of niacin, 2.22 mg of pyridoxine hydrochloride, 6.66 mg of calcium pantothenate, 44 μg of biotin, 200 μg of folic acid, 3 μg of vitamin B_{12}, and 2.22 mg of thiamine hydrochloride per 100 gm diet.

At the end of 28 days, growth had ceased in both groups of animals, at which time each group of 25 rats was divided into 5 sub-groups, consisting of 5 rats each, which received an oral supplement of riboflavin to give the following levels: 0.5, 1.0, 1.5, 2.0, and 4.0 μg/gm food. The group receiving 4.0 μg/gm food was used as a positive control in each temperature group on the assumption that it was receiving a

[3] Purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

level of riboflavin adequate for maximum growth. Riboflavin was mixed into the food slurry daily. The rats were allowed to eat ad libitum and refusals were weighed three times per week.

During cold exposure, the symptoms described previously (Vaughan and Vaughan, '57) were observed. Two rats died during cold exposure.[4] These deaths were attributed to excessive losses of blood caused by animals chewing at the necrotic tissue of their tails. The repletion experiment was continued for 28 days, at the end of which all rats were placed on a complete stock diet [5] and recovered satisfactorily at both environmental temperatures.

RESULTS AND DISCUSSION

Growth. It should be noted that the cold rats were not as heavy as the warm rats at the beginning of the experiment. This was due, in large part, to the initial delay in the cold-induced acceleration of food intake. In order to determine whether initial weight modified subsequent weight gains, within-group correlations of these variables were calculated. There was no consistent correlation between initial weight and subsequent gain. Consequently, the unadjusted weight gains were used in the succeeding statistical analyses.

The mean weight changes for the 28-day experimental period are summarized in table 1. The "p" values presented in this table are the results of "t" tests of the simple effects between sub-groups, for which the estimate of error was derived from an analysis of variance of the complete data. Tests of simple effects were indicated because of the presence of interaction between diet and temperature factors for each criterion.

[4] Missing data were treated by substitution of the sub-group mean. Conservatism was preserved by subtracting two degrees of freedom from those associated with the error term, according to convention.

[5] Friskies, Albers Milling Company, Division of Carnation Company, Los Angeles, California.

TABLE 1

Weight and food intake response of rats receiving graded levels of riboflavin at 5° and 25°C

(28-day experiment)

RIBOFLAVIN INTAKE		Δ W			ADJUSTED FOOD INTAKE [1]			FINAL ADJUSTED WEIGHT [2]	
		5°	p [3]	25°	5°	Mean [4]	25°	5°	25°
μg/gm		*gm*		*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
0.5		− 4	ns	− 6	453	(352)	251	215	275
	p [5]	< 0.01		< 0.01		< 0.05		ns	ns
1.0		+ 31	ns	+ 34	530	(436)	341	226	289
	p	< 0.01		< 0.01		< 0.05		ns	< 0.05
1.5		+ 63	ns	+ 73	620	(498)	376	232	318
	p	ns		< 0.1		ns		ns	ns
2.0		+ 77	ns	+ 88	626	(514)	401	244	325
	p	ns		< 0.1		ns		< 0.05	ns
4.0		+ 63	< 0.01	+ 100	631	(530)	430	229	328

[1] Adjusted for regression on initial weight.

[2] Adjusted for regression on initial weight and food intake.

[3] Significance of weight changes between 5°C and 25°C.

[4] No interaction. Mean adjusted intakes used for test of single effects.

[5] Significance of changes between levels of riboflavin.

The growth response of rats receiving the same level of riboflavin in the diet was not affected by the environmental temperature when the riboflavin level was 2.0 μg/gm or less. At 4.0 μg/gm, however, the growth of the warm rats was significantly greater than that of the corresponding cold rats.

The results reported here are somewhat in variance with published reports. Mitchell et al. ('50), on the basis of experiments with growing pigs, believe that the riboflavin requirement per unit of food is greater at 5° than at 29°C. Ershoff ('52) reported impaired survival and depressed growth in rats subjected to a cold (2°C) environment. The latter experiment is not strictly comparable to ours, however, since Ershoff's animals were already deficient when placed in the cold room, while ours were allowed to become deficient at 5°C, a procedure which allows the animals to become adjusted to cold before being subjected to the additional stress of the deficiency. As the results show, the relation of the riboflavin requirement to food intake is not invalidated by cold exposure, under the conditions of this experiment.

It will be seen from table 1 that there is a significant dosage effect of riboflavin on growth up to the 1.5 μg/gm level. Above that level, growth increments are relatively small. However, there is a significant increase in growth between the 1.5 μg/gm and 4.0 μg/gm levels in the warm rats. In view of the diminishing growth increments, it was decided to plot the weight differences against the logarithm of the dosage level (fig. 1). If the intersections of the log dose lines (calculated) with the lines of maximum growth are taken to be the minimum requirements for riboflavin, the requirement for maximum growth of the warm rats becomes approximately 2.3 μg/gm and that of the cold rats 2.2 μg/gm of food.

Food intake. Food intakes were treated by a covariance analysis, in which the observed intakes were adjusted in terms of their regression on the initial weights of the rats.[6]

[6] $b = 0.901$; $r_{xz} = 0.53$, where x = initial weight and z = food intake.

These adjusted mean intakes may be seen in table 1. Both the level of riboflavin in the food and cold exposure had a significant effect on the food intake and there was no significant interaction between these effects. Food intake rises rapidly with increasing levels of riboflavin until the 1.5 μg/gm level is reached, whereupon the increments become quite small. The general pattern is quite similar to that shown by

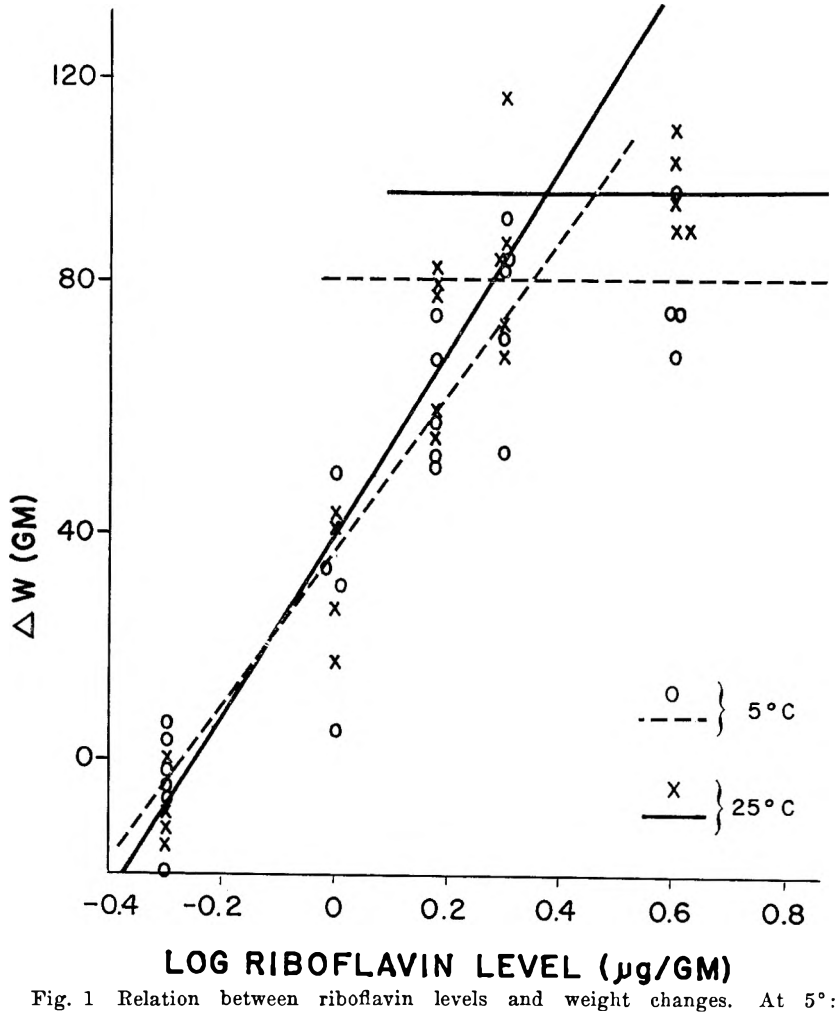

Fig. 1 Relation between riboflavin levels and weight changes. At 5°: $Y = 36.0 + 137.2X$; 25°: $Y = 40.2 + 162.1X$.

the growth response curve, and breaks at the same level of riboflavin intake. It is thus quite evident that a considerable degree of inanition occurs during riboflavin deficiency in rats at both 5° and 25°C.

The differences in food intake between cold and warm rats are quite uniform across successive levels of riboflavin, as shown by the lack of significant interaction variance. It thus appears that, as with a thiamine deficiency (Vaughan and Vaughan, '57), the portion of the appetite stimulated by the cold exposure is not affected by the anorexia induced by the riboflavin deficiency.

Since experimental rats were allowed to eat ad libitum and since several statements in the literature concerning the efficiency of food utilization of riboflavin-deficient rats have been based on paired feeding experiments (Sure, '41; Sure and Dichek, '41; Voris et al., '42), the food intake and growth data were analyzed by a multiple covariance analysis, in which final weight was adjusted for food intake and initial weight.[7] These values are presented in table 1 and represent the most probable final weights which would have occurred on each level of riboflavin, had the animals weighed the same initially and eaten the same amount of food during the experiment.

As expected, the rats held at 25°C used their food more efficiently for growth. In addition, the multivariate analysis shows that the level of riboflavin had a significant effect on the adjusted final weights, indicating that the efficiency of food utilization for growth, as well as food intake, is lowered in riboflavin-deficient rats at both temperatures.

It is a general opinion that the efficiency of food utilization for growth is the most critical factor in limiting the growth of riboflavin-deficient rats. In our opinion, the results presented in table 1 do not support this view. Food intake in both cold and warm rats is depressed profoundly in the deficient rats: by 28% and 42% at 0.5 μg/gm in the cold and

[7] $b_1 = 0.701$; $b_2 = 0.300$; $R_{y.xz} = 0.87$; $r_{xz} = 0.53$; $r_{yz} = 0.72$; $r_{xy} = 0.79$, where x = initial weight, y = final weight, and z = food intake.

warm rooms, respectively. A comparison of the maximum decrements in the unadjusted weights (— 81 gm at 5°C and —106 gm at 25°C) with the maximum decrements in the adjusted weights (— 29 gm at 5°C and — 53 gm at 25°C) indicates that the observed weight changes are far from being accounted for by adjusting the final weights for differences in food intake and initial weight. It is our opinion that food intake is at least as important as the efficiency of food utilization in determining the growth response of rats to riboflavin.

SUMMARY

Weight changes and food intakes were measured in rats kept at two environmental temperatures and receiving adequate and suboptimal levels of riboflavin in the diet.

There were no significant differences in weight loss or gain between the rats held at 25°C and 5°C when the level of riboflavin was 2.0 μg/gm food or less.

Data obtained on the food intake of these animals suggest that the growth response to various levels of riboflavin is a function of both appetite and efficiency of food utilization and that the cold-induced intake increment remains constant at all levels of riboflavin intake.

ACKNOWLEDGMENT

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NUTRITION STUDIES IN THE COLD

III. EFFECTS OF COLD ENVIRONMENT ON "CHOLESTEROL" FATTY LIVERS [1]

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The production of "cholesterol" fatty livers has been described by a number of investigators. Best, Channon and Ridout ('34) reported that 2% cholesterol in a diet containing 40% fat caused a marked increase in the esterified cholesterol fraction of the liver lipides. Aylward, Channon and Wilkinson ('35) reported that the above ratio of cholesterol: dietary fat produced an increase in the esterified cholesterol of the liver within 4 hours after ingestion of the diet and concluded that this increase was the initial stage in the production of "cholesterol" fatty livers. Loizides ('38) observed an increase in the liver content of esterified cholesterol by feeding high-fat diets alone. Ridout et al. ('52) reported that cholesterol deposition in the liver increased with prolonged feeding times.

Sellers and You ('49, '52) found that the total liver lipides were decreased when rats were fed a hypolipotropic diet in a cold environment. This observation has been confirmed and extended by studies in our laboratory. In a previous report (Treadwell, Flick and Vahouny, '57) it was shown that cold functioned as an effective lipotropic agent over a

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wide range of dietary fat levels in both immature and young adult male rats. Later, it was reported (Treadwell, Flick and Vahouny, '58) that cold also cured fatty livers previously induced by feeding low-protein, high-fat diets which were low in the known dietary lipotropic factors.

From these findings, it was considered of interest to determine first, the effect of cold on the production of "cholesterol" fatty livers and secondly, the curative effect of a cold environment on animals in which cholesterol fatty livers had been previously induced. Data are also presented on the growth, food intake, calorie intake, blood cholesterol levels at weekly intervals during the feeding of diets with and without cholesterol, and the changes brought about by cold on the various fractions of the liver lipides.

EXPERIMENTAL

The two diets, 9 and 10, employed in the present study are shown in table 1, and are numbered consecutively to the diets used previously (Treadwell et al., '57). Casein was included in the control diet 9 and experimental diet 10 at the 20%

TABLE 1

Percentage composition of diets

CONSTITUENTS	DIET 9	DIET 10
	%	%
Casein [1]	20	20
Salt mixture [2]	5	5
Celluflour [3]	2	2
Starch	25	24
Sucrose	25	24
Vitamin mixture [4]	2	2
Lard	18	18
Cod liver oil	2	2
Sodium taurocholate (U.S.P.)	1	1
Cholesterol (U.S.P.)	0	2

[1] Vitamin-free casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Hubbell, Mendel and Wakeman ('37).
[3] Purified cellulose, Chicago Dietetic Supply House, Chicago, Ill.
[4] See Treadwell, Flick and Vahouny ('57).

level, and both diets contained 1% sodium taurocholate to facilitate efficient absorption of the dietary cholesterol. The fat content of each diet was 20%. Choline and inositol were omitted from the diets so that the methionine content of casein was the only known source of lipotropic substances.

White male rats of the Carworth strain, weighing 100 to 150 gm were used. Pre-experimental and experimental treatments of the animals have been described previously (Treadwell et al., '57). Prior to feeding the experimental diets, tail-blood samples were taken from randomly selected rats for the determination of cholesterol fractions. Nine rats were placed on each of the diets at 1°C for 21 days; two additional groups of 24 rats each were placed on the same diets at 25°C for the first 21-day experimental period. Blood samples were drawn from 8 rats in each group at both temperatures at 7-day intervals for analysis of total and free cholesterol. At the end of this first period, the animals at 1°, and 8 animals on each diet at 25° were sacrificed while in the post-absorptive state. The livers were removed, blotted, weighed, homogenized, and extracted for total lipides as described by Dury and Treadwell ('53). Experiment I was designed to study the effect of a cold environment on the production of "fat" and "cholesterol" type fatty livers and is designated the preventive study. Of the 16 animals remaining on diets 9 and 10 at 25°, 8 from each group were continued on their respective diets at 25°, while the others were transferred to 1° and continued on the same diets. These 4 groups of rats were maintained for a second 21-day experimental period prior to sacrifice and extraction of the livers for total liver lipides. The second phase was designed to study the effect of cold on previously induced fatty livers, and is designated the curative study period.

Total liver lipides were determined gravimetrically by the method of Dury and Treadwell ('53). The liver lipide fractions were determined on a portion of the original lipide extract. A modification of the method of Fiske and Subbarow ('25) was used for determination of lipide phosphorus.

Phospholipide values were obtained by the conventional method (lipide p $\times$ 25). Liver and blood cholesterol fractions were determined by the method of Sperry and Webb ('50). Triglyceride levels were calculated by subtracting the combined values of phospholipide, free cholesterol and esterified cholesterol (calculated as oleate) from the value for total liver lipides.

Apparent differences between groups were analyzed for significance by the "t" test of Fisher ('38), and only those showing a P value less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Food consumption, caloric intake, and growth at 25° and 1°. As was discussed in the initial study of this series (Treadwell et al., '57), the use of a non-conductor on the floor of the cages in the cold greatly increased the percentage survival of the animals subjected to the cold environment. In experiment I, the survival on diet 9 at 1° was 100%, while on diet 10 at 1°, 87% of the animals completed the initial 21-day study. Essentially the same survival was found in experiment II at 1°. All animals at room temperature in both experiments survived.

Although the food and caloric intake was greater on both diets at 1° than at 25°, as shown in table 2, the gain in weight in the cold was considerably less than at room temperature. This relationship was reported previously by Sellers and You ('49) and by this laboratory (Treadwell et al., '57). The animals at 25° on diets 9 and 10 had higher protein efficiency ratios than at 1°, indicating a more efficient utilization of protein for growth at room temperature. The 1% cholesterol in diet 10 had no effect on the protein efficiency ratio at either 25° or 1°.

In the second 21-day experimental period the animals which were continued at room temperature maintained their food and caloric intake, but the rate of growth and utilization of dietary protein for growth decreased. The rats trans-

TABLE 2

Food intake, weight gain, and protein efficiency ratios at 25° and 1°C [1]

DIET NO.	TEMPERATURE	NO. OF RATS	FOOD	CALORIES/ DAY	WEIGHT GAIN	PROTEIN EFFICIENCY RATIO [2]
			gm/day		*gm/day*	
			Experiment I, preventive study			
9	25°	24	11.6 ± 0.2	53.3 ± 1.5	3.8 ± 0.2	1.6 ± 0.1
9	1°	9	14.4 ± 0.7	66.3 ± 3.1	1.6 ± 0.3	0.5 ± 0.1
10	25°	24	12.6 ± 0.3	59.3 ± 1.4	4.2 ± 0.1	1.7 ± 0.0
10	1°	7	16.3 ± 1.1	76.7 ± 1.7	2.1 ± 0.4	0.6 ± 0.1
			Experiment II, curative study			
9	25°	8	13.1 ± 0.4	60.3 ± 2.0	2.6 ± 0.2	1.0 ± 0.1
9	1°	8	16.8 ± 1.0	77.5 ± 1.4	0.6 ± 0.2	0.2 ± 0.1
10	25°	8	13.1 ± 0.4	61.8 ± 2.1	2.8 ± 0.2	1.1 ± 0.1
10	1°	7	17.5 ± 0.4	82.0 ± 1.9	0.4 ± 0.2	0.1 ± 0.1

[1] The figures represent the mean ± standard error.

[2] Grams change in weight/gm protein ingested.

ferred to the cold on both diets exhibited a substantial increase in food intake and a marked reduction in growth. These changes led to very low values for the protein efficiency ratio which suggest that very little protein was utilized for growth in these animals at 1°.

Liver weight, total liver lipides, and lipide fractions. Data on liver weight and total liver lipides for experiments I and II are shown in table 3. At the end of the initial period, the livers of rats on diet 10 at 1° and at 25° were significantly heavier than those of the animals on diet 9. The "cholesterol" fatty livers of rats on diet 10 were macroscopically characterized by appearing pale yellow and were friable in contrast to normal tissue. The lipide content of these livers was markedly greater than of the livers of rats on control diet 9. The liver lipides of cold-exposed animals on diet 9 were significantly lower than in those on this diet at 25° as had been shown previously (Treadwell et al., '57), and which indicates a lipotropic effect of cold. In contrast, however, was the finding that the livers of animals on diet 10 at both temperatures contained the same amount of total liver fat. The significance of these data will be discussed below.

After the curative period (experiment II), liver weights, expressed in terms of body weight, were essentially unchanged from the initial weights, and these values were comparable at 1° and 25° on each diet. Continuation of the rats on diet 9 at 25° produced a further elevation of liver lipides, whereas exposure of these animals to cold caused a reduction in the level. These results confirm previous work in this laboratory (Treadwell et al., '58) on the curative effect of cold on "fat" fatty livers. Continuation of the rats on diet 10 at room temperature resulted in an increase in total liver lipides, which was more marked than with diet 9. In the cold, however, liver lipides were even more markedly elevated than at 25°, indicating a qualitative difference in the effect of cold on the two types of fatty livers.

The liver lipide partition showed a number of interesting differences between the fat- and cholesterol-type fatty livers.

TABLE 3

Liver weights and total lipides[1]

DIET NO.	TEMPERATURE, C	NO. OF RATS	LIVER WEIGHT		TOTAL LIPIDES	
			gm	*gm/100 gm B.W.*	*% of liver*	*gm/100 gm B.W.*
			Experiment I, preventive study			
9	25°	8	9.9 ± 0.5	4.8 ± 0.1	9.9 ± 0.7	475 ± 30
9	1°	9	7.3 ± 0.4	4.5 ± 0.1	6.0 ± 0.4	271 ± 13
10	25°	8	13.6 ± 0.9	6.3 ± 0.3	19.1 ± 1.8	1,174 ± 88
10	1°	7	10.2 ± 0.6	5.8 ± 0.1	19.5 ± 0.5	1,126 ± 33
			Experiment II, curative study			
9	25°	8	11.9 ± 0.5	4.4 ± 0.2	12.6 ± 0.2	584 ± 83
9	1°	8	9.9 ± 0.3	4.6 ± 0.1	7.1 ± 0.3	324 ± 18
10	25°	8	17.2 ± 0.9	6.2 ± 0.2	24.4 ± 0.9	1,514 ± 83
10	1°	7	14.8 ± 0.6	6.5 ± 0.2	26.7 ± 0.8	1,730 ± 78

[1] The figures represent the mean ± standard error.

As is shown in figure 1, the liver lipides of rats on diet 9 at 25° were composed largely of triglycerides, 56%, and phospholipides, 31%. In the cold-exposed animals on the same diet, the lower level of liver fat contained one-third the percentage of triglyceride and twice the level of phospholipide as the lipides of the 25° rats. This relationship is in accord with the concept that cold-exposed rats metabolize

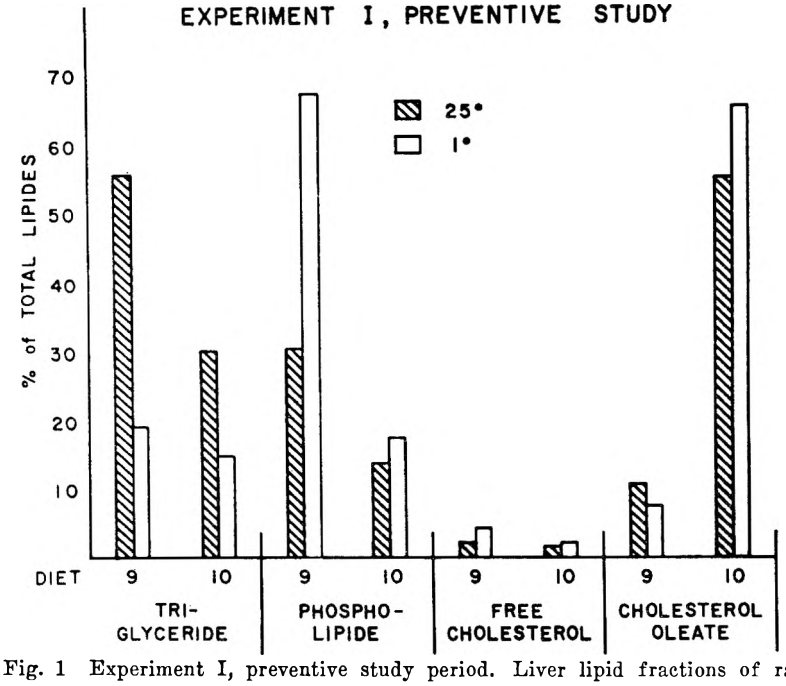

Fig. 1 Experiment I, preventive study period. Liver lipid fractions of rats at 25° and 1° fed low-sterol diet 9, or diet 10 with 1% cholesterol for 21 days.

dietary fat more efficiently than animals at room temperature, and this effect appears to be due to an increased metabolism of triglycerides with a concomitant increase in phospholipide formation. In contrast to the lipide partition in the "fat" fatty livers, the liver lipides of the cholesterol-fed rats contained a smaller percentage of triglycerides and phospholipides, with a corresponding greater concentration of hepatic cholesterol, primarily the esterified form. Again,

the comparable animals on diet 10 at 1° displayed a markedly lower percentage of liver triglycerides and an increase in the phospholipide fraction. Total liver lipides were not reduced by cold, however, because of an even higher esterified cholesterol level at 1° than at 25°.

Figure 2 shows the effects of continuation of animals with fat- and cholesterol-fatty livers at 25°. An additional three

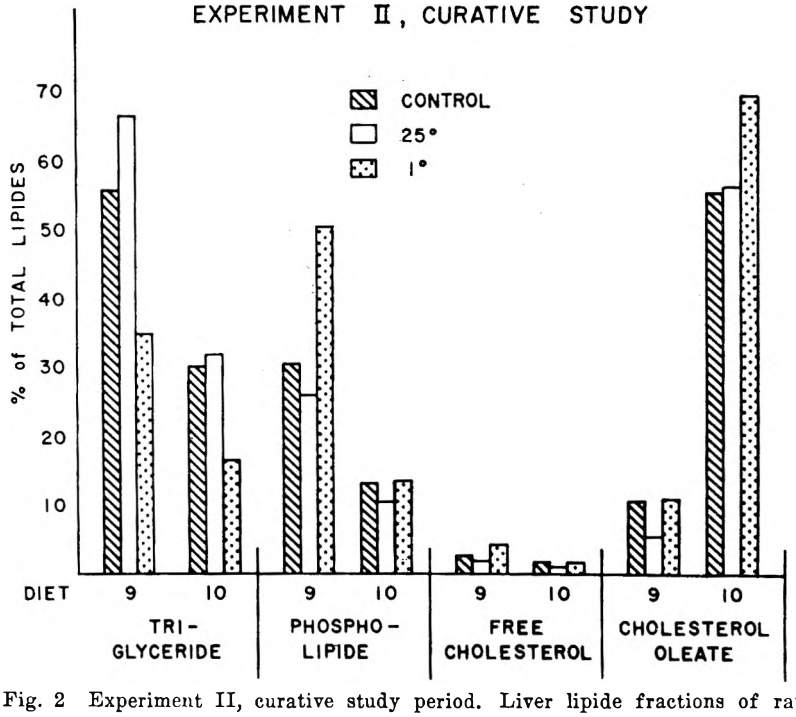

Fig. 2 Experiment II, curative study period. Liver lipide fractions of rats at 25° and 1° fed low-sterol diet 9, or 1% cholesterol diet 10 for 21 days after development of fatty livers. Control values represent the liver lipide fractions of rats fed diets 9 and 10 at 25° for 21 days prior to experiment II.

weeks on diet 9 at 25° did not markedly alter the liver lipide partition; however, at 1° there was an increase of phospholipides and a marked reduction in liver triglycerides resulting in a lower overall hepatic fat content. Thus, cold had a curative effect on the "fat" fatty liver.

Continuation of animals on diet 10 at 25° led to a further increase in total liver lipide (see table 3). Analysis of the lipide fractions showed no alteration in the percentage composition of the liver lipide. When placed at 1°, the increase in liver fat was even greater than at room temperature. However, as is shown in figure 2, cold again resulted in a marked reduction of the triglyceride level of liver, substantiating the results of the first experiment and clearly demonstrating the lipotropic effect of cold on the triglyceride fraction. The increase in the level of phospholipides was not as marked as with diet 9 at 1° and accounted for only a small fraction of the elevated total liver lipides. There was also no change in the free cholesterol fraction at 1° on diet 10, but the esterified form comprised 68% of the liver lipides as compared to 56% at room temperature.

The data of experiment II show clearly that cold exhibits a lipotropic effect in prevention and regression of fat-fatty livers, and that this effect is principally on the triglyceride fraction of liver lipides. Conversely, a cold environment has no effect on the production of "cholesterol" fatty livers, but acts in an anti-lipotropic manner with previously-induced "cholesterol" fatty livers, i.e., gives further increases in total liver lipides. This increase is due to a markedly increased level of esterified cholesterol which compensated for the reduction in the triglyceride fraction by cold. An explanation for these differences in liver cholesterol levels is suggested by a consideration of the cholesterol fractions of blood during the preventive and curative periods (figure 3). Changes in the blood cholesterol levels of animals on diet 9 at both temperatures were insignificant in both experiments. On diet 10, cholesterol levels at both temperatures were markedly increased during the initial period, but those at 1° were considerably higher than those at 25°, attaining a level of almost 260 mg % after two weeks. Also in experiment II, total cholesterol levels at 1° were significantly higher than at 25° at the termination of the experimental period. As shown in figure 3, differences in the levels of

total cholesterol were due primarily to changes in the esterified fraction, as were the changes in liver cholesterol.

From these data it appears that cold has an effect on cholesterol metabolism which is independent of its effects on the triglyceride and phospholipide fractions of total liver

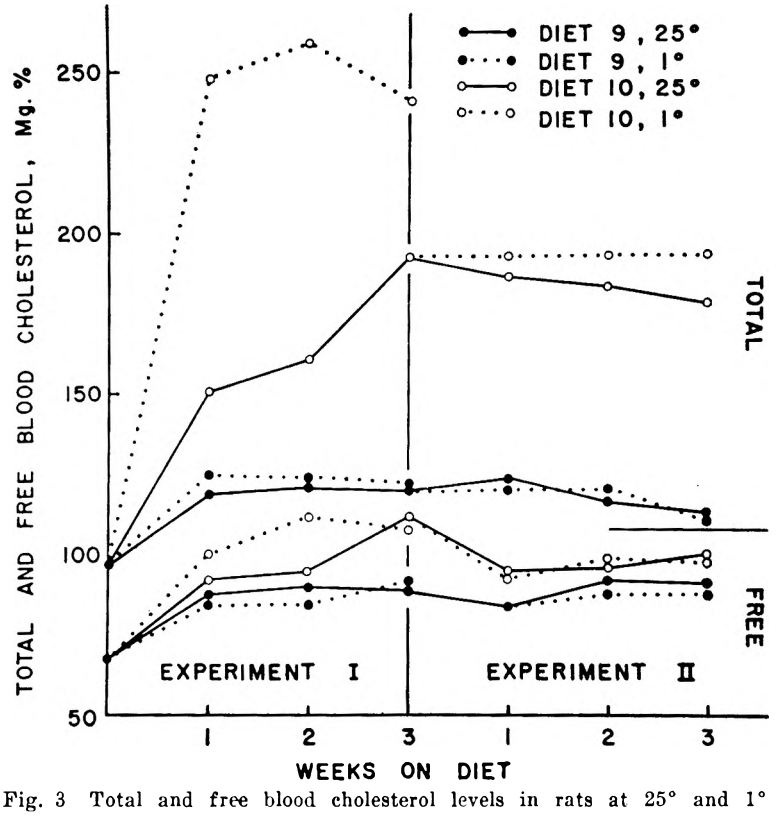

Fig. 3 Total and free blood cholesterol levels in rats at 25° and 1° fed low-sterol diet 9, or 1% cholesterol diet 10 for 21 days in experiment I, the preventive study, and for 21 additional days in experiment II, the curative study.

lipides. While the cold environment had a consistent lipotropic influence on hepatic triglycerides, there was a simultaneous increase in blood and liver esterified cholesterol levels in rats fed a cholesterol-fat diet. Since a comparable deposition of cholesterol was not found in animals on the low-sterol diet, it is possible that cold had an effect on

cholesterol absorption from the intestinal tract. Another possibility to be considered is that normal metabolism of cholesterol in the liver is impaired in the cold, leading to a greater accumulation of esterified cholesterol at 1° than at room temperature.

SUMMARY

Young male rats received hypolipotropic diets containing 20% protein and 20% fat, with and without 1% cholesterol. Comparable groups were maintained at 25° and 1°C. Rats on the low-cholesterol diet at 25° had a marked accumulation of hepatic triglycerides during the initial 21-day period, while at 1°, triglyceride increase was not observed. Thus, cold exhibited a preventive lipotropic effect on "fat" fatty livers. Cholesterol-fed rats at 25° developed yellow fatty livers characterized by a high level of esterified cholesterol. Similar animals at 1° had even greater increases in liver lipides due to a further elevation of the esterified cholesterol level, while the triglyceride fraction was reduced by cold.

During a second 21-day curative study, rats with "fat"' and "cholesterol" fatty livers were maintained at 25° or transferred to 1°. Cold had a curative lipotropic action on hepatic triglycerides in rats on both diets. Livers of rats on the low-sterol diet at 1° had a normal level and distribution of liver lipide fractions, while in cholesterol-fed rats, cholesterol esters accumulated further at both temperatures. The effect was more pronounced at 1°.

Thus, cold had both preventive and curative lipotropic effects on "fat" fatty livers, and an independent "anti-lipotropic" action on the "cholesterol" fatty liver in rats.

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