RESORPTION OF EMBRYOS IN RATS ON LATHYRUS ODORATUS DIET

DONALD G. WALKER[1] AND ZOLTON T. WIRTSCHAFTER
The Research Laboratory of the Medical Service, Veterans Administration Hospital, and the University of Oregon Medical School, Portland

SEVENTEEN FIGURES

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The age of the experimental animal at which Lathyrus odoratus feedings are started determines the rate of development and degree of severity of lathyrism. Geiger, Steenbock and Parsons ('33), who produced lathyrism in both young (two-month-old) and adult rats, were among the first investigators to demonstrate that the signs of lathyrism were manifested sooner and became decidedly more pronounced in young rats in comparison with adults treated for the same periods. The most crippling deformities of the thorax and vertebral column were commonly seen in the young rats. In a more recent report on the lesions of the skeleton and other mesodermal tissues in rats fed Lathyrus odoratus seeds, Ponseti and Shepard ('54) showed that the marked differences observed depended upon the age of the experimental animals. When the feedings of the lathyrus diet were begun the rats in the younger group were 22 days of age and those of the oldest group were one year old. Thus, dissecting aneurysms of the aorta were observed only in the younger group; in fact, "no aneurysms were produced when feedings had been started later than 51 days of age."

From the foregoing it is evident that the earlier the age of the animal at the beginning of lathyrus feedings the more fulminating is the observed metabolic disorder. To investi-

[1]Present address: The Department of Anatomy, Johns Hopkins Medical School, Baltimore, Maryland.

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gate further this important age-intensity relationship it was decided to expose simultaneously the youngest possible experimental mammals, namely the embryos, and the maternal adult to the lathyrus factor. From the day of conception the pregnant rats were maintained on a pea-pellet diet until autopsy at varying intervals during the gestation period.

METHODS

Environment. An air-conditioned room in which the temperature was regulated at 73 to 75°F. and the relative humidity at 45 to 50% housed the experimental animals. Refilling of water bottles and food cups was done at the same time each day. The shavings in the cages were replaced every third day.

Diet. The diet for the control animals consisted of: whole wheat 67.5, casein 15.0, whole milk powder 10.0, iodized NaCl 0.75, $CaCO_3$ 1.5 and hydrogenated vegetable oil 5.25%. A concentrate of fish oil in an amount to give 1.5 chick units of vitamin D and 10.5 U.S.P. units of vitamin A per gram was included in this diet.

A mixture in the proportions of one part each of the control ration and seeds of the pea, Lathyrus odoratus, constituted the experimental diet which was administered to the adult female rats throughout selected periods after the determined day of conception. Not only was it necessary to grind the peas into a fine powder before mixing with the control diet, but as an added precaution this 1:1 mixture was made up into hard pellets and designated "pea-pellet diet." Early in the experiment it was observed that rats placed on the powdered form of the experimental diet tended to reject the pea fragments in the mash and for this reason the experimental pea diet was supplied in hard pellet form.

Animals. Sixty-nine adult female rats of the Long-Evans strain were used in this experiment. By means of the vaginal smear technic the day of conception was determined and time of parturition calculated from this initial finding. Eighteen normal animals were maintained on the control

ration from time of conception until day of autopsy which varied at daily intervals from the 11th to the 21st day of gestation. Fifty-one experimental rats were maintained on the "pea-pellet diet" for selected periods after the time of conception. No group contained less than 4 animals.

Vaginal smears were examined microscopically daily from time of conception to the day of implantation. Invariably in experimental as well as in normal control rats the presence of red blood cells, denoting implantation, were observed on the 12th or 13th day of gestation.

All animals were roentgenographed at weekly intervals throughout gestation in search for radiological evidence of osseous changes.

Autopsy. All autopsy procedures were performed with rats under ether anaesthesia, and a detailed inspection was made of all viscera. Particular attention was focused upon the uterus. To allow comparative quantitative evaluation of the resorptive process, color photographs were made of the uterus, placentae and embryos.

Because no photographs depicting the successive stages of embryonic development of the normal rat from the time of implantation (12th day after conception) to the termination of gestation (22nd day after conception) were available, such a record was compiled in our laboratory (figs. 2 to 9). These normal embryos were removed by Caesarian section, photographed and then fixed directly in either absolute alcohol or 10% neutralized formol in anticipation of further study histologically. To avoid undue distortion only a few of the early embryos were removed from their amniotic envelopes. The uniformity of form, size and color of embryos at each successive age is impressive.

RESULTS

It became evident at the autopsy examination of the first experimental animals that the embryos were in various stages of resorption. This pathological process was characterized by selective involvement and appeared to be in a random

pattern. This lack of uniformity brought about by the resorptive process is in direct contrast to the regularity in form and size of embryos of a normal litter.

That the extent of variation in the resorptive process may be extreme is evidenced in the case of the animal (W425) which was treated with the experimental diet from the 15th to 23rd day after conception (fig. 17). Two non-viable but full-sized embryos were removed from the uterus which showed 10 cicatricial plaques demarking sites of previous implantation. The extremes of the resorptive process, therefore, may be encountered simultaneously in a single pregnant animal.

The intensity of the total resorptive process of a uterus must be expressed in terms of the extent to which each embryo has been affected. Therefore, the following classification was devised and applied to each individual embryo in a litter: Grade I: the embryo is not viable and subcutaneous edema and even slight maceration may be evident. However, the crown-rump length of each embryo must not be less than that of the viable embryos; grade II: all embryos are diminished in size and markedly macerated; grade III: resorption of all embryos is complete. All placentae are present though diminished in size; grade IV: embryos and placentae are completely resorbed. The cicatrized plaques persist for several weeks as physiological implantation markers at the former sites of the placentae (figs. 10–13).

A graphic correlation of the grades of resorption disclosed at autopsy was compiled with the number of days between conception and the termination of the pea-pellet diet as the independent variable (fig. 1).

The weekly roentgenograms of non-pregnant females showed osseous changes on and after the 14th day of receiving the pea-pellet diet. However, the pregnant females showed no osseous changes throughout the normal gestation period (22 days). Severe osseous pathology of these pregnant females was observed on the 28th day after conception and the initiation of the pea-pellet diet.

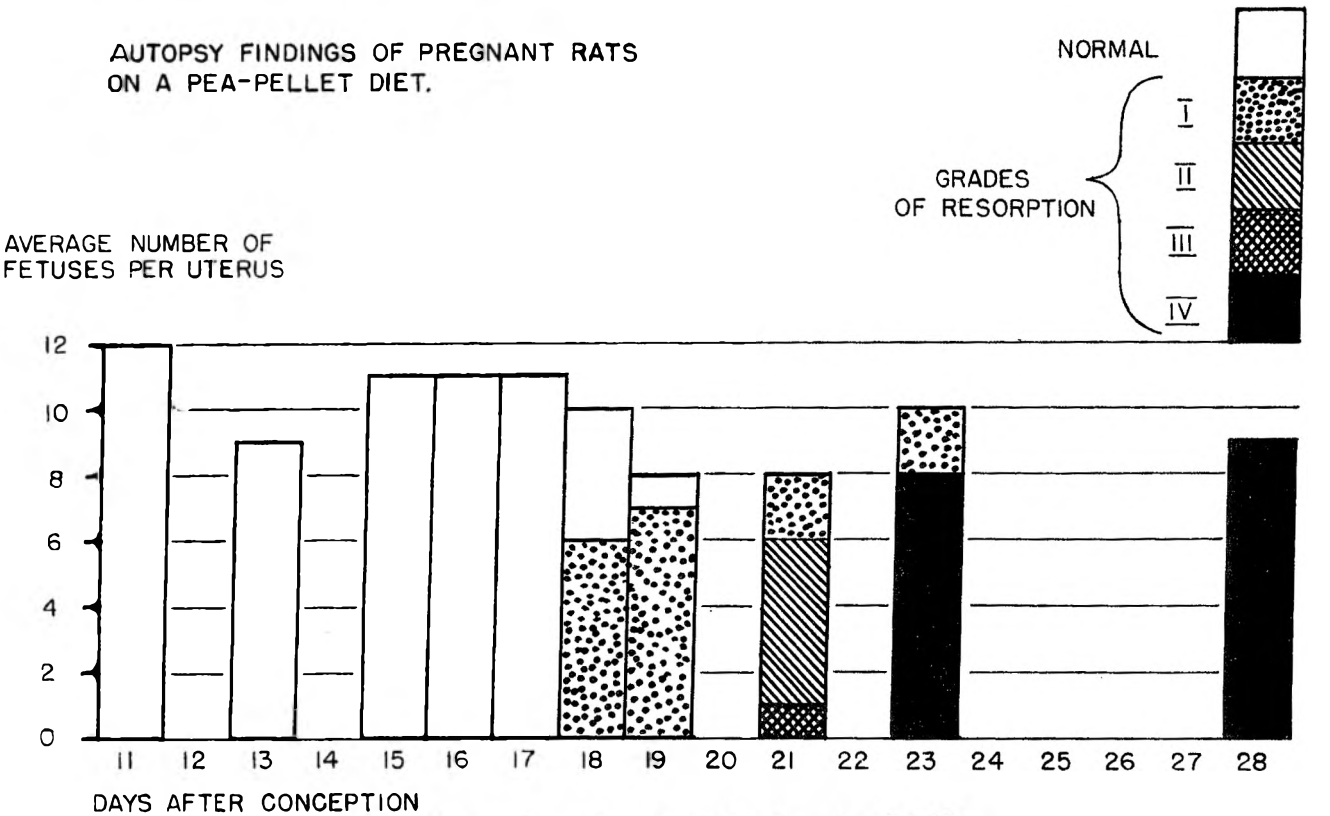

Fig. 1 Autopsy findings of pregnant rats on a pea-pellet diet.

There was no instance of resorption in experimental animals autopsied on or before the 16th day after conception. In fact, animals fed the experimental diet continually from the day after conception until autopsy on the 16th day had embryos all of which were uniform in size and form, and crown-rump lengths normal for the respective embryonic age.

The resorptive process was first encountered in experimental animals treated with the lathyrus pea diet for periods of one week or longer prior to autopsy on the 18th day of gestation. In this experimental group the average size of the "litter" was 10 embryos, 4 of which were classified as normal and 6 as "grade I" of resorption.

Experimental animals treated for periods of a week or longer prior to autopsy on the 19th day of gestation possessed on the average 8 embryos of which one was normal and 7 were classified as "grade I" of resorption.

Experimental animals treated for periods of a week or longer prior to autopsy on the 21st day after conception had an average of 8 embryos all of which were undergoing resorption and classified as follows: two as "grade I," 5 as "grade II," and one as "grade III" of resorption.

In the single experimental animal (W425) treated for one week prior to autopsy on the 23rd day after conception, two non-viable but full-sized embryos were removed from the uterus which demonstrated 10 cicatricial plaques demarking sites of previous implantations. The two embryos were classified as "grade I" and, of course, the plaques denote "grade IV" of resorption.

Experimental animals maintained on the pea-pellet diet for periods of from one week after conception until the day of autopsy one week after the calculated time of parturition showed on the average, 9 cicatricial plaques, the evidences of former implantations, classified as "grade IV" of resorption.

The osseous lesions characteristic of adult lathyrism did not appear in the presence of a normal placenta.

SUMMARY

An analysis has been based on the autopsy findings of rats placed on an experimental pea-pellet diet for varying numbers of days of the gestation period and maintained until autopsy. The period between conception and termination of a pea-pellet diet determined the grade of resorption of the rat embryo. The young experimental mammal, the rat embryo, cannot survive in the presence of the lathyrus factor beyond the 16th day of gestation. In contrast, the adult female exposed quantitatively to the same toxic pea factor demonstrates, during gestation, no osseous or other lesions characteristic of lathyrism. Only when fed the pea-pellet diet for periods longer than that of gestation (22 days in the rat) do any osseous lesions appear, although in non-pregnant females they appear by the 14th day of treatment.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Dean C. Altman, the head of the Photographic Department of this hospital, and Mr. Bruce Stuart of his staff for their helpful assistance.

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PLATE 1

EXPLANATION OF FIGURES

Uniformity of form and size of embryos of the normal "litter" is striking at every stage of development.

2 Normal rat embryos 11 days after conception.

3 Normal rat embryos 13 days after conception.

4 Normal rat embryos 16 days after conception.

5 Normal rat embryos 17 days after conception.

6 Normal rat embryos 18 days after conception.

7 Normal rat embryos 19 days after conception.

8 Normal rat embryos 20 days after conception.

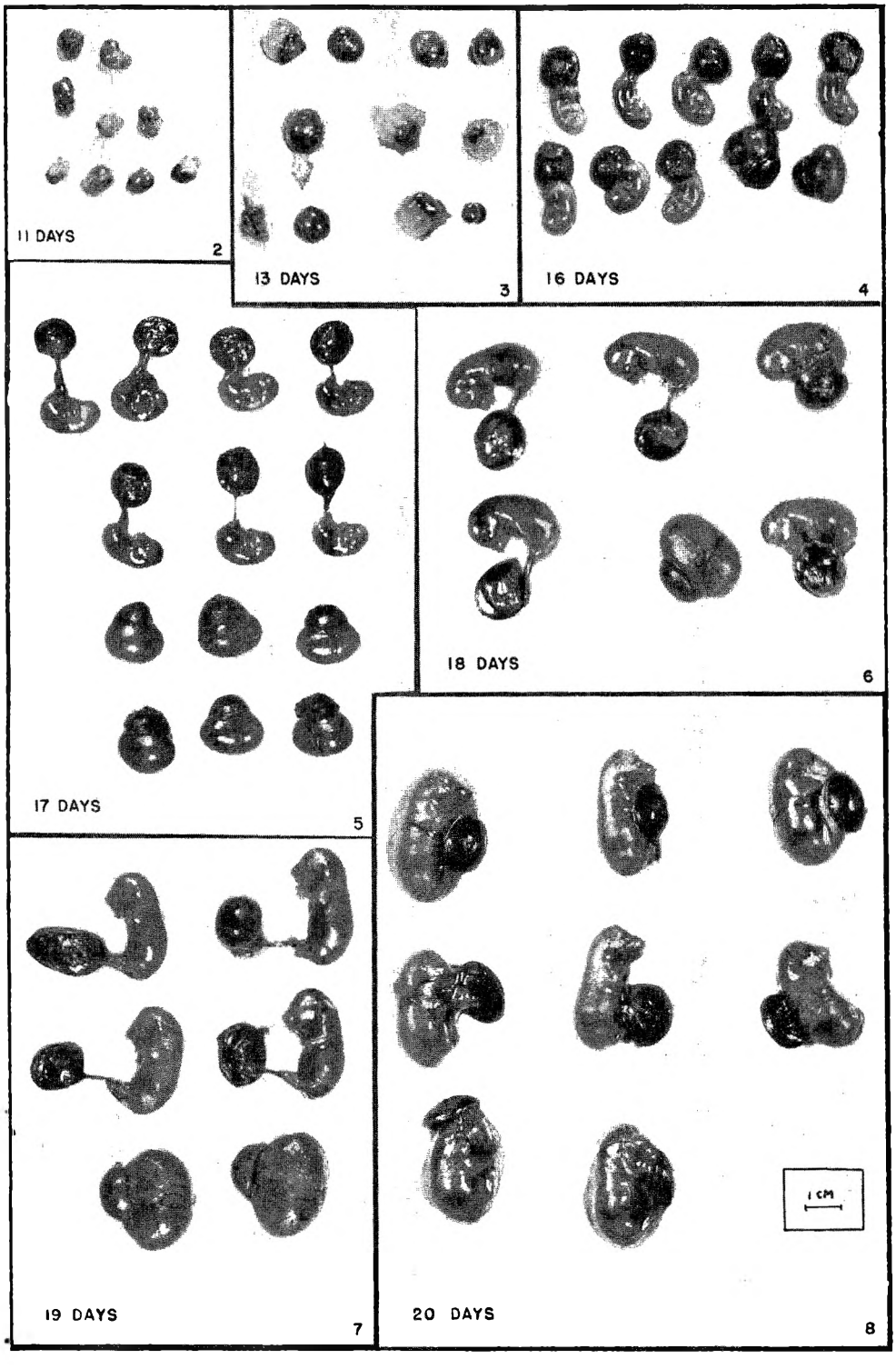

11 DAYS
2
13 DAYS
3
16 DAYS
4
17 DAYS
5
18 DAYS
6
19 DAYS
7
20 DAYS
8
1 CM

PLATE 2

EXPLANATION OF FIGURES

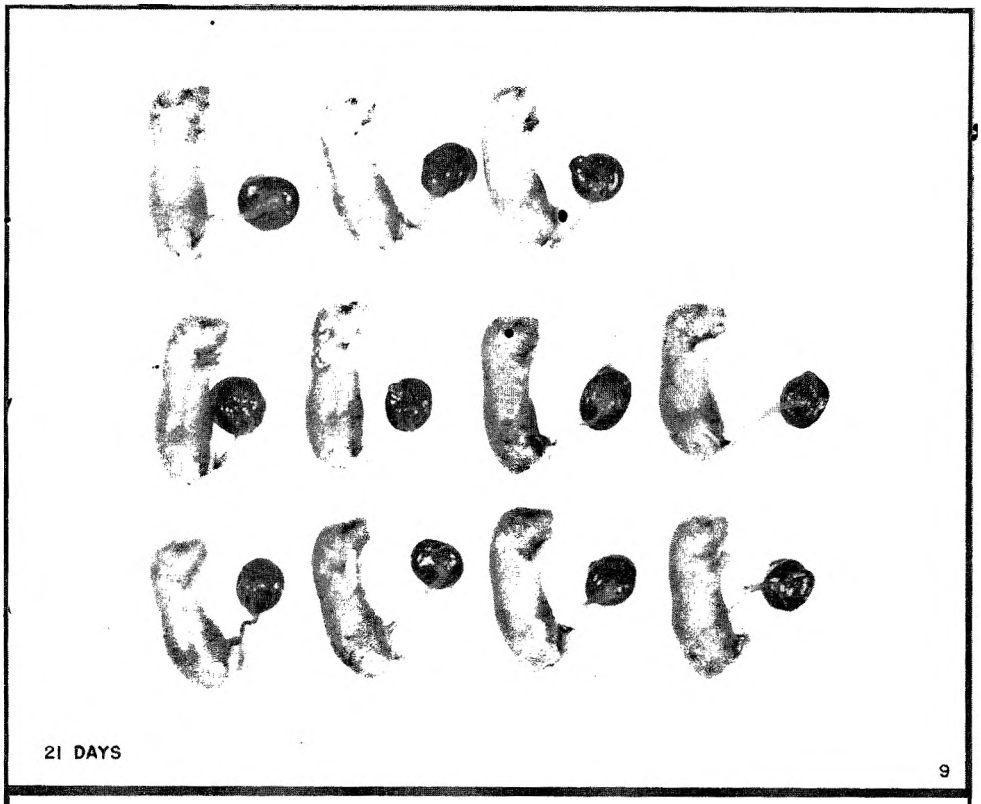
9 Normal rat embryos 21 days of age after conception (the day before birth).
10 to 13 Grades of resorption:

Grade I: The crown-rump length of each embryo must be within normal limits. However, the embryo is not viable and subcutaneous edema and even maceration may be evident.

Grade II: All embryos are diminished in size and markedly macerated.

Grade III: Resorption of all embryos is complete. All placentae are present though diminished in size.

Grade IV: The cicatrized uterine plaques represent the final stage of the resorptive process. These physiologic implantation markers persist for several weeks even after complete resorption of the embryos and placentae.


21 DAYS
9

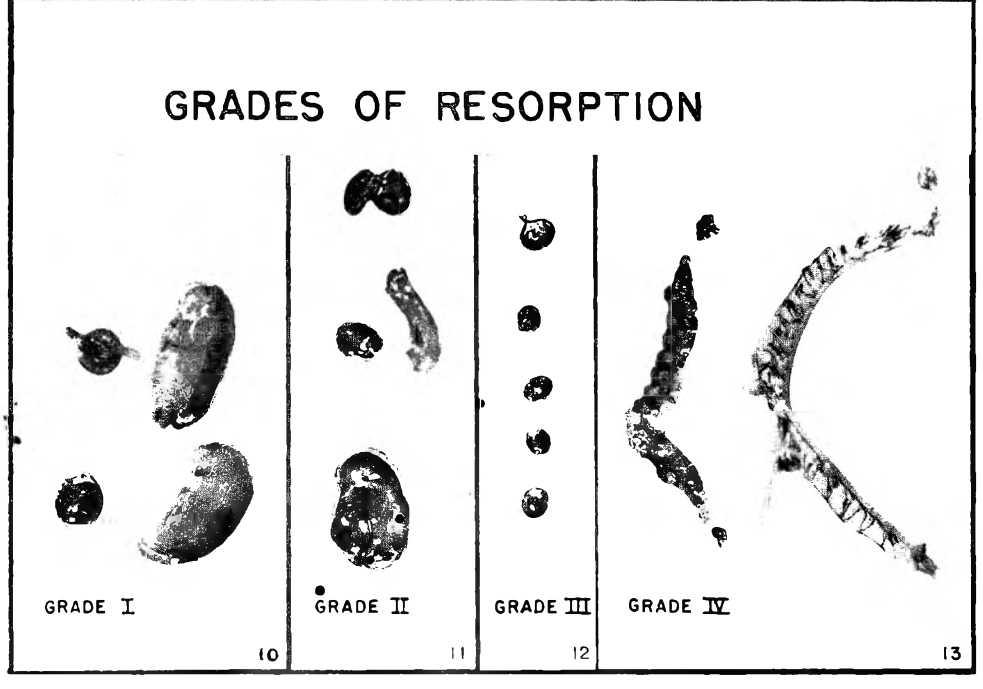

GRADES OF RESORPTION
GRADE I
10
GRADE II
11
GRADE III
12
GRADE IV
13

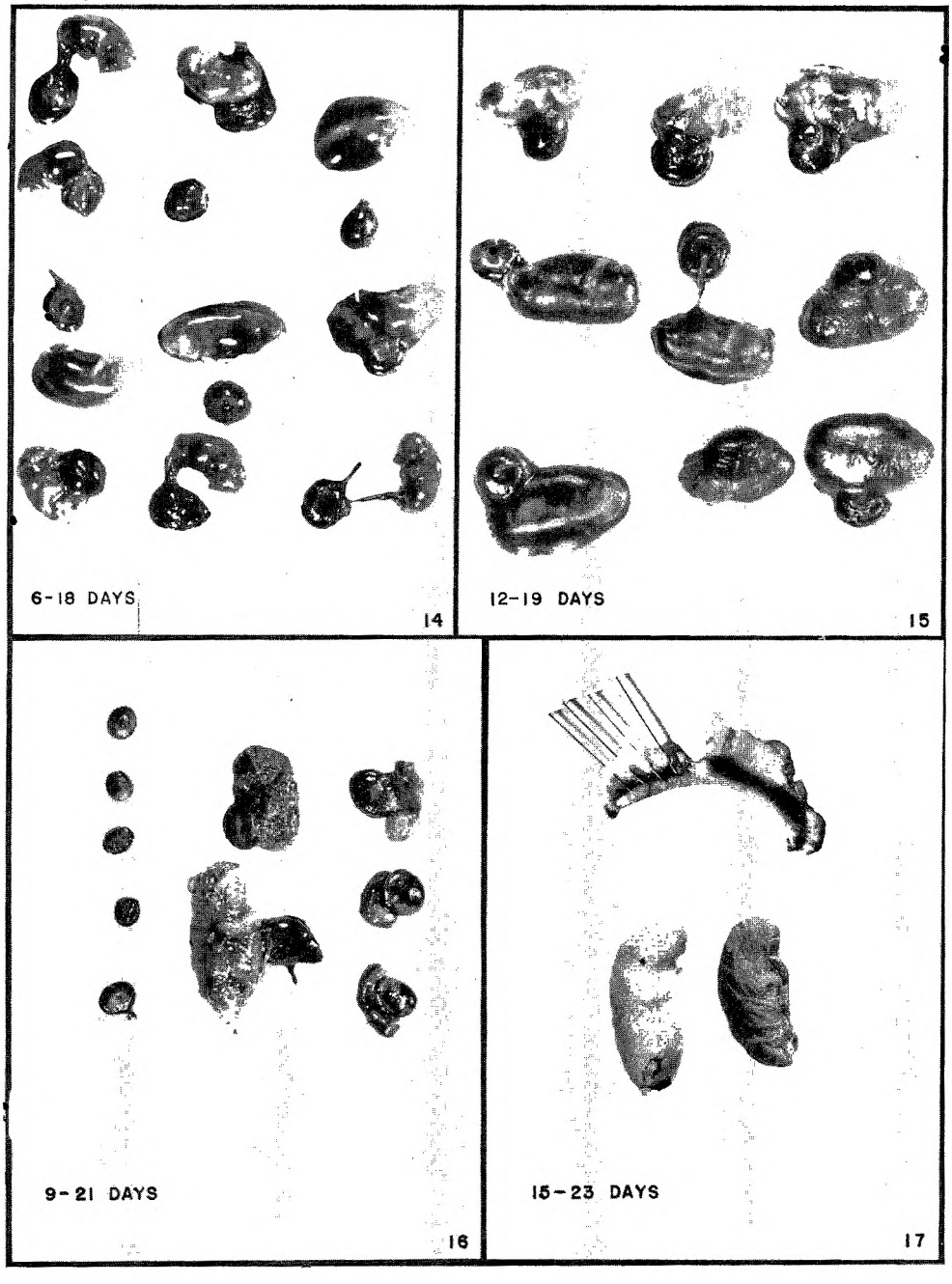
PLATE 3

EXPLANATION OF FIGURES

The variation with regard to the degree of involvement and the random pattern of selection of the uterine resorptive process is seen in these findings at autopsy of 4 pregnant female rats fed the pea-pellet diet between 6–18, 12–19, 9–21, and 15–23 days after conception, respectively.

FIGURE	PERIOD ON THE PEA-PELLET DIET (DAYS AFTER CONCEPTION)	NORMAL EMBRYOS	GRADES OF RESORPTION			
			I	II	III	IV
14	6–18	5	5			
15	12–19		9			
16	9–21		1	4	5	
17	15–23		2			8

In this manner, the uterine resorptive process may be expressed in terms of the degree to which each embryo is involved.


6-18 DAYS
14
12-19 DAYS
15
9-21 DAYS
16
15-23 DAYS
17

ESTROGENIC INHIBITION OF FETAL RESORPTION IN LATHYRISM

DONALD G. WALKER[1] AND ZOLTON T. WIRTSCHAFTER
The Research Laboratory of the Medical Service, Veterans Administration Hospital, and the University of Oregon Medical School, Portland

SIXTEEN FIGURES

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That no rat embryo can survive in the presence of the lathyrus factor was demonstrated by feeding a pea diet daily to pregnant rats as late as the 16th day after conception (Walker and Wirtschafter, '56). When the uterus of such experimental animals is examined daily throughout gestation one encounters resorption of the embryos, 18 days of age and older. Even when the lathyrus diet was started on the day of conception, no resorptions were seen before 18 days of gestation and invariably resorption was total and complete by 28 days after conception.

In contradistinction, Nelson, Asling and Evans ('52) found that "instituting a deficiency of pteroylglutamic acid in the rat as late as 9 days after breeding invariably resulted in fetal death (resorption) . . . Normal young resulted when the deficiency was not started until 15 days after breeding."

In a study of pregnancy in pyridoxine-deficient rats, Nelson, Lyons and Evans ('53) report that "with 21 days of deficiency prior to breeding, the uninjected group showed 94% resorptions with early appearance of erythrocytes in the vaginal smear (day 10) due to early resorbing sites."

Thus, it is apparent that in states of deficiency of either pyridoxine or folic acid, resorptions occur early in gestation, in fact, even before the normal time of implantation (12th

[1]Present address: The Department of Anatomy, Johns Hopkins Medical School, Baltimore, Maryland.

day after conception). However, resorption induced by the lathyrus factor occurs late in gestation only after the 17th day of gestation.

The lack of uniformity of the lathyrus-induced resorptive process as encountered in the latter days of gestation requires a macroscopic classification that expresses the intensity of the total uterine resorptive process in terms of the extent to which each embryo has been affected. Therefore, to each individual embryo in a litter we applied the classification stated in the preceding paper from this laboratory.

The estrogenic hormones afford marked protection against "early" resorption of embryos by prolonged pyridoxine deficiency (Nelson, Lyons and Evans, '53). Studies were, therefore, instituted to evaluate the effect of estrone and progesterone on the "late" resorption induced in pregnant rats by the lathyrus factor of the pea-pellet diet.

METHODS AND PROCEDURES

Our environment, diet, and autopsy procedures were identical to those described in the preceding paper.

Animals. Of the 20 adult female rats of the Long-Evans strain used in this experiment, 5 were uninjected controls and 15 were injected with estrogens. The times of conception and implantation were determined by the vaginal smear technic. Roentgenograms of each rat were made at 7, 14 and 21 days after conception.

RESULTS

All embryos undergo resorption when pregnant rats are fed the pea-pellet diet. This uterine resorptive process is characterized by selective involvement of embryos in a random order. Although there were 44 sites of implantation in the animals of this uninjected control group, autopsy at 21 days after conception disclosed 17 embryos of grade I, 19 of grade II, 5 of grade III, 3 of grade IV of resorption, respectively, and not a single *normal* fetus (table 1 and plate 1).

TABLE 1

EFFECT OF ESTROGENS ON UTERINE RESORPTIONS IN RATS FED THE PEA-PELLET DIET[1] THROUGHOUT GESTATION

TREATMENT	IDENTIFICATION NUMBER OF PREGNANT RATS	NUMBER OF IMPLANTATION SITES	STATUS OF EMBRYOS AND PLACENTAE				
			NORMAL	GRADES OF RESORPTION			
GROUP, DOSAGE, AND ROUTE OF INJECTION				I	II	III	IV
UNINJECTED CONTROLS	43	6		4	2		
	416	5		4		1	
	548	8		2	5	1	
	560	9		2	6	1	
	618	8		3	3	2	
	656	8		2	3		3
	TOTAL	44		17	19	5	3
DISTRIBUTION IN PER CENT				39	43	11	7
10MG. OF ESTRONE[2] INTRAPERITONEALLY INJECTED DAILY FROM DAY 1 UNTIL AUTOPSY ON DAY 21 OF GESTATION.	64	8		1	4	3	
	403	7	3	3	1		
	638	10	1	9			
	680	7		4	1	2	
	810	9		2	7		
	TOTAL	41	4	19	13	5	
DISTRIBUTION IN PER CENT			10	46	32	12	
4MG. OF PROGESTERONE[3] SUBCUTANEOUSLY INJECTED DAILY FROM DAY 1 UNTIL AUTOPSY ON DAY 21 OF GESTATION.	603	9	2	1	5	1	
	614	9	5	2			2
	658	11	2	2	4	3	
	671	9	5			2	2
	TOTAL	38	14	5	9	6	4
DISTRIBUTION IN PER CENT			37	13	24	16	10
10MG. OF ESTRONE INTRAPERITONEALLY INJECTED AND 4MG. OF PROGESTERONE SUBCUTANEOUSLY INJECTED DAILY FROM DAY 1 UNTIL AUTOPSY ON DAY 21 OF GESTATION.	73	10	7	2	1		
	286	11	4	2		5	
	540	5	4		1		
	593	7	4		2		
	622	10	7		3		
	663	7	6	1			
	TOTAL	50	32	5	7	5	
DISTRIBUT ON IN PER CENT			64	10	14	10	

Five pregnant rats fed the pea-pellet diet were injected with 10 μg of estrone, daily, from day one to day 21 after conception. Of 41 sites of implantation disclosed at autopsy 37 were undergoing resorption and classified as follows: 19 embryos of grade I, 13 of grade II, 5 of grade III of resorption and 4 normal fetuses (plate 2).

A second experimental group consisted of 4 pregnant rats, which were fed the pea-pellet diet and injected with 4 mg of progesterone, daily, from day one to day 21 of gestation. Of the 38 sites of implantation evident at autopsy 24 were involved in the resorptive process as follows: 5 embryos were of grade I, 9 of grade II, 6 of grade III, 4 of grade IV, and 14 were entirely normal (plate 3).

In a third experimental group 6 rats fed the pea-pellet diet were injected both with 10 μg of estrone and 4 mg of progesterone, daily, from day one to day 21 of gestation. Of 50 implantations disclosed at autopsy only 18 were undergoing resorption. The latter were classified as follows: 5 embryos of grade I, 7 of grade II and 5 of grade III of resorption. That the daily administration of estrone and progesterone inhibited the fetal resorption induced by a factor in the Lathyrus pea is evidenced by the presence of 32 normal fetuses (plate 4).

SUMMARY AND CONCLUSION

A resorptive process intercepts fetal development on the 18th day of gestation when pregnant rats are fed a pea-pellet diet. The estrogenic hormones, particularly when administered in combination, afford protection against the lathyrus factor responsible for resorption.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Dean C. Altman, the head of the Photographic Department of this hospital, and Mr. Bruce Stuart of his staff for their helpful assistance.

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PLATE 1

EXPLANATION OF FIGURES

Macroscopic photographs of the uterine contents of uninjected pregnant rats treated with the pea-pellet diet from the day of conception until autopsy, 21 days later. All embryos are undergoing resorption. (See table 1, also.)

GH656	1	GH560	2
B 548	3	GH618	4

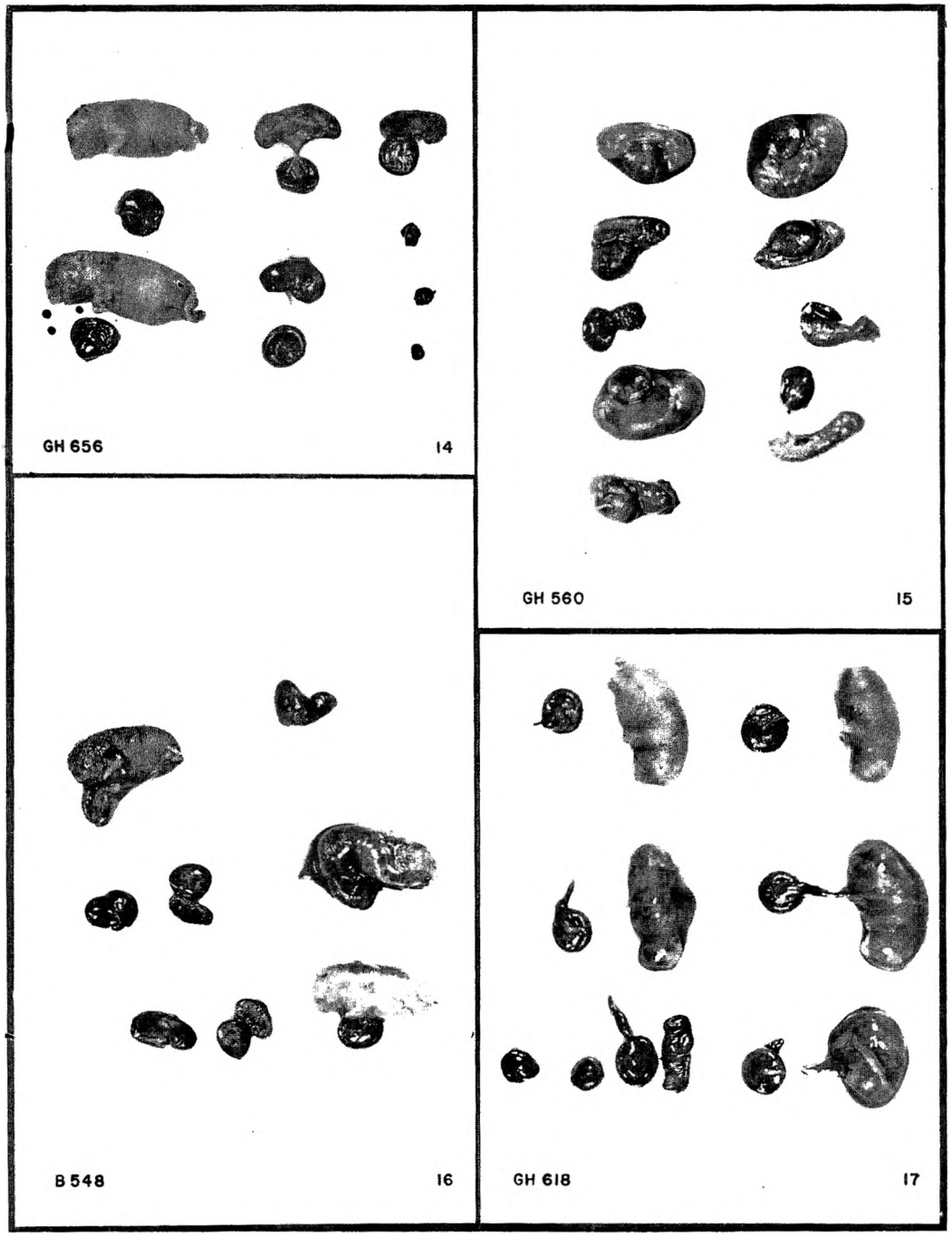

GH 656
14
GH 560
15
B 548
16
GH 618
17

PLATE 2

EXPLANATION OF FIGURES

Macroscopic photographs of the uterine contents of estrone-injected pregnant rats treated with the pea-pellet diet from the day of conception until autopsy, 21 days later. A small proportion of embryos appeared normal, whereas the majority are undergoing resorption.

BH403	5	G810	6
GH680	7	B 64	8

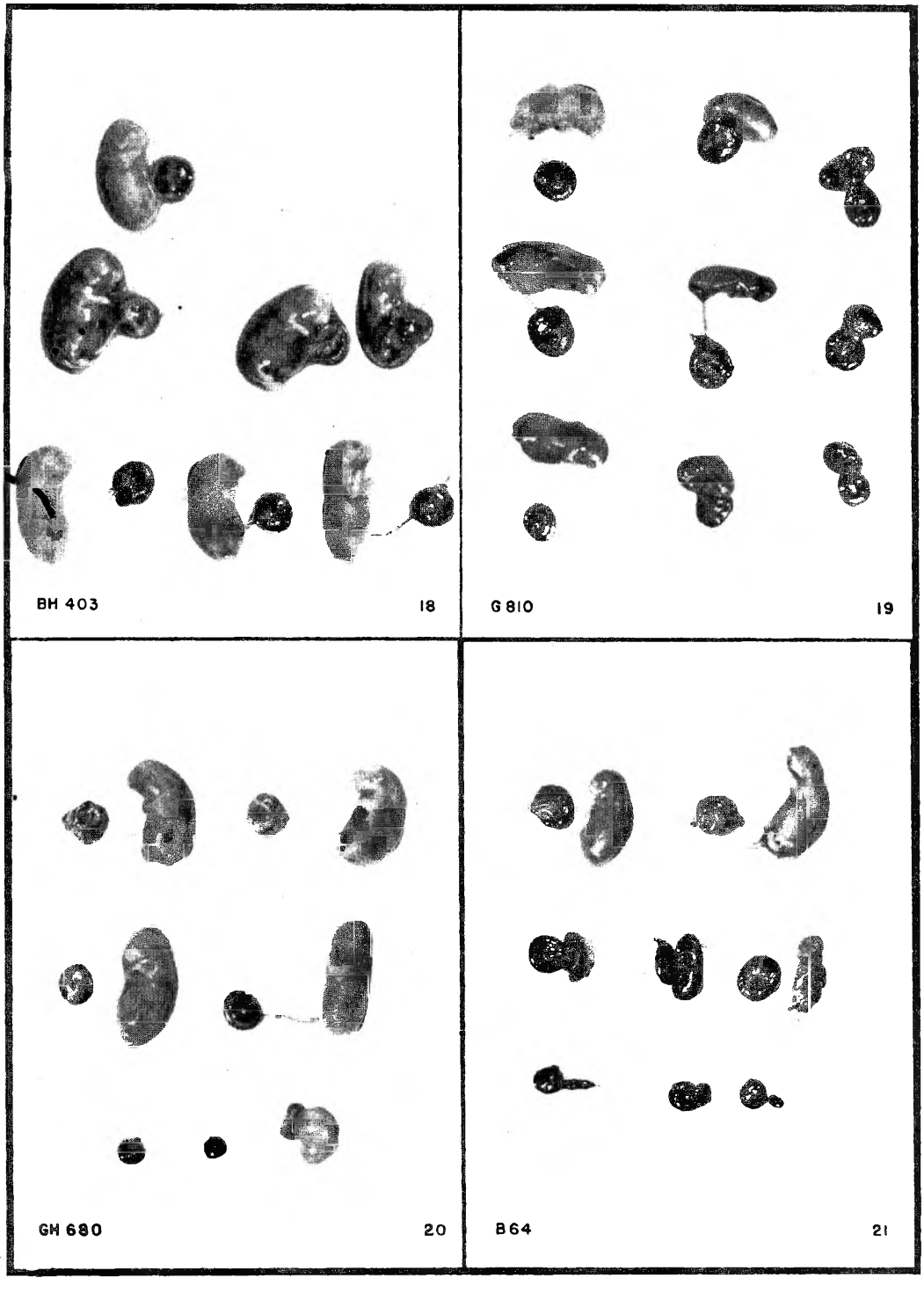

BH 403
18
G 810
19
GH 680
20
B64
21

PLATE 3

EXPLANATION OF FIGURES

Macroscopic photographs of the uterine contents of the progesterone-injected pregnant rats treated with the pea-pellet diet from the day of conception until autopsy, 21 days later. A large proportion of the embryos of each pregnant rat are normal.

GH614	9	GH658	10
GH671	11	B 603	12

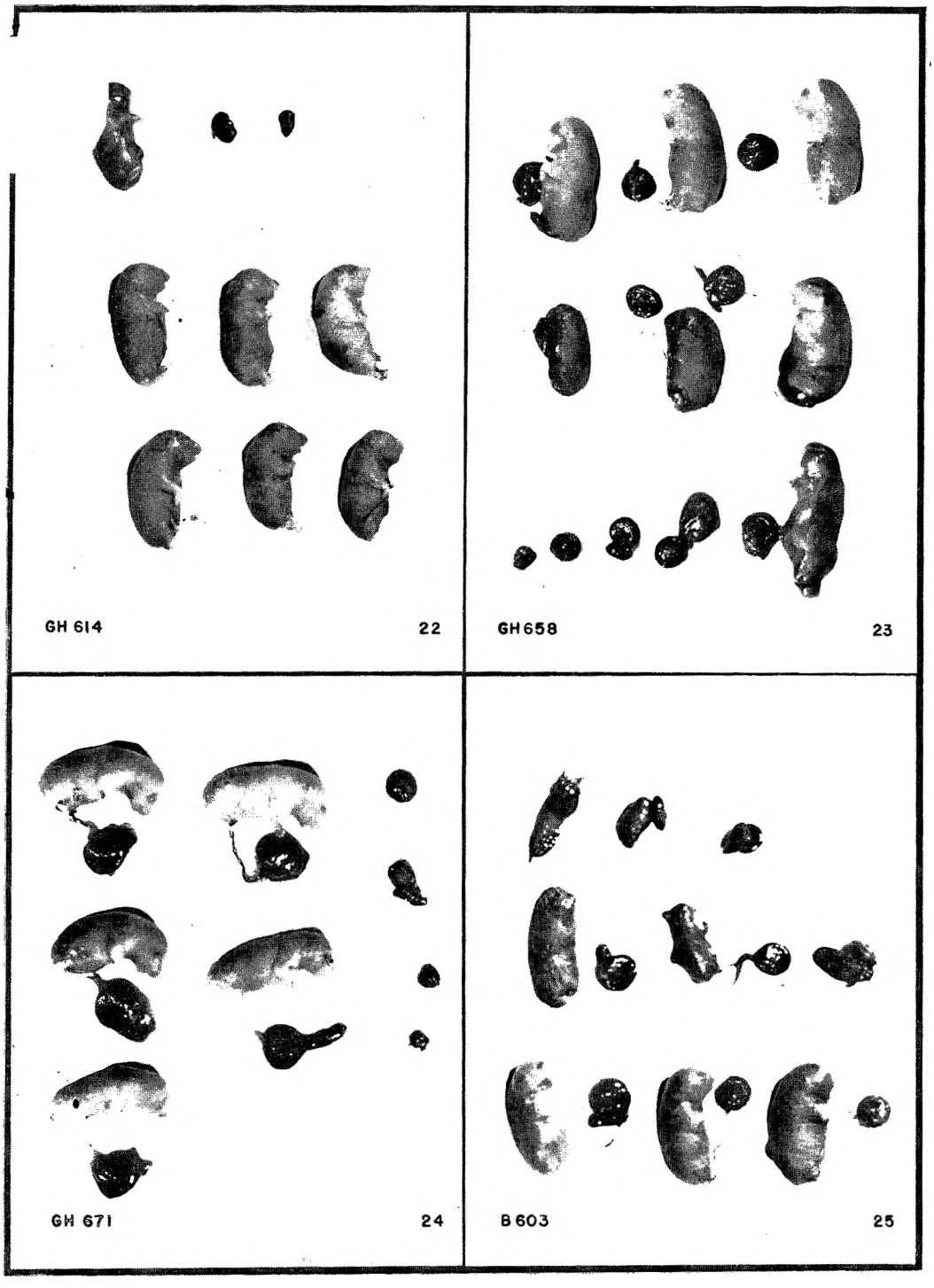
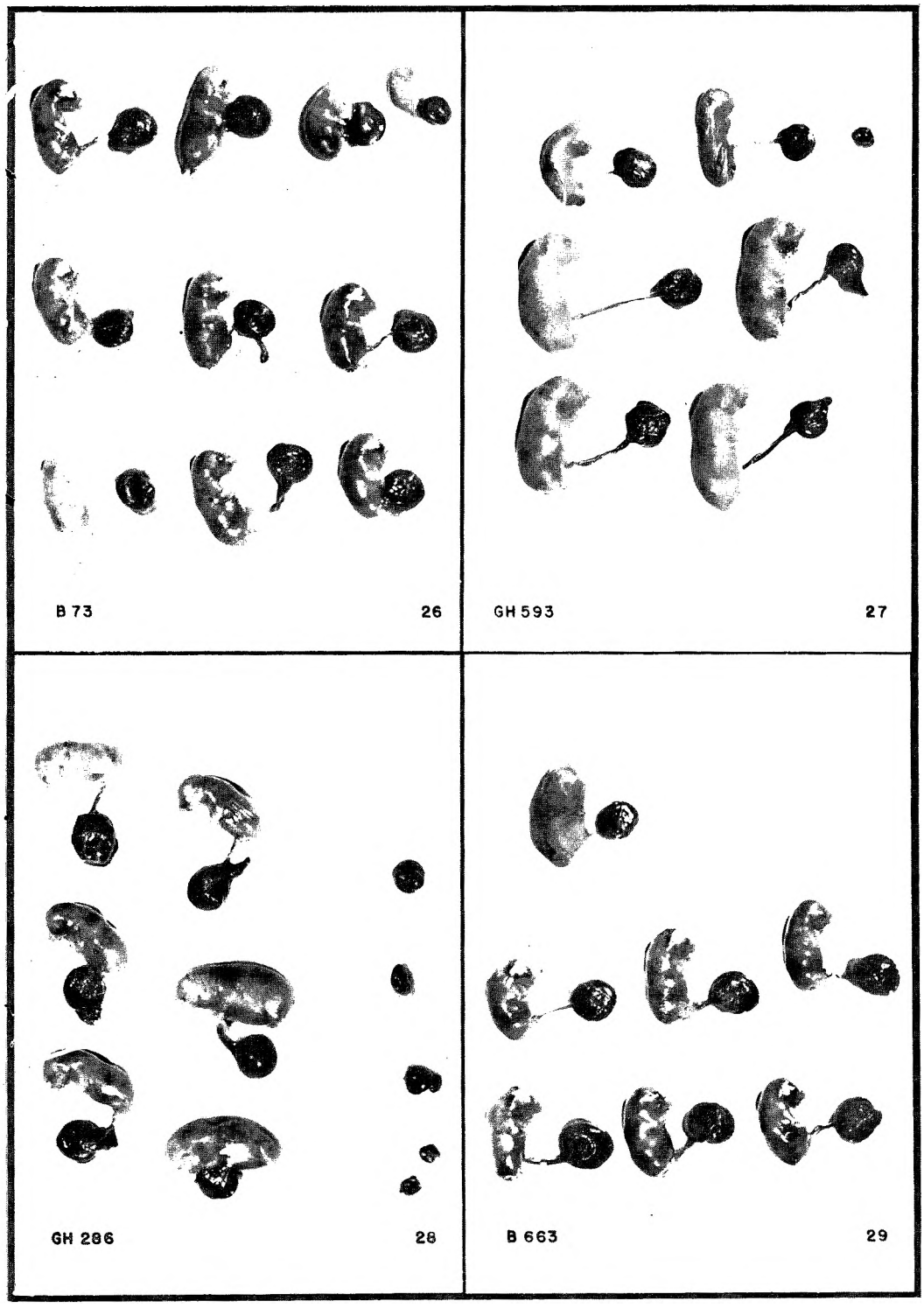

GH 614
22
GH 658
23
GH 671
24
B 603
25

PLATE 4

EXPLANATION OF FIGURES

Macroscopic photographs of the uterine contents of pregnant rats injected with both estrone and progesterone while on the pea-pellet diet from the day of conception until autopsy, 21 days later. The majority of the embryos are normal and viable.

B 73	13	GII593	14
GII286	15	B 663	16


B 73
26
GH 593
27
GH 286
28
B 663
29

THE EFFECT OF LOW TEMPERATURE AND DIETARY CALCIUM UPON MAGNESIUM REQUIREMENT [1]

D. M. HEGSTED, J. J. VITALE AND HELEN McGRATH
Department of Nutrition, Harvard School of Public Health and Department of Biochemistry, Harvard Medical School, Boston, Massachusetts

FOUR FIGURES

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During recent years there have been rather few studies upon the essential nutrient magnesium. Duckworth ('38–'39) reviewed the rather extensive literature available at that time and concluded, in part that "A principal effect of magnesium deficiency appears to be a disturbance of normal calcium metabolism." The principal observations supporting this conclusion include marked increases in the calcium content of heart, muscle, and particularly kidney tissue of magnesium-deficient rats (Tufts and Greenberg, '37–'38a), and extensive deposits of calcium salts in various tissues in deficient calves (Moore et al., '36), including calcification of the yellow elastic fibres of the endocardium, the large arteries, the aorta, and jugular vein. Tufts and Greenberg ('37–'38b) also observed that a high calcium content of the diet increased the severity of magnesium deficiency and raised the magnesium requirement. This observation may possibly explain the apparent development of magnesium deficiency in milk-fed calves (Duncan et al., '35) at relatively high levels of magnesium intake.

[1] Supported in part by grants-in-aid from the National Institute of Arthritis and Metabolism (A-194)C, National Institutes of Health, Public Health Service, Bethesda, Maryland; J. M. Kaplan Fund, Inc., New York; General Mills, Inc., and the Nutrition Foundation, New York.

During recent years Constant and Phillips ('52) hav studied the "calcinosis syndrome" in the cotton rat. The development of this condition appears to be partly but not entirely dependent upon the magnesium intake. The pathological changes are at least reminiscent of the "stiffness syndrome" in the guinea pig (van Wagtendonk and Wulzen, '50). It may be significant that the latter species apparently has an unusually high magnesium requirement (Roine et al., '49) and the diets used to produce the stiffness syndrome are high in calcium.

The classical syndrome of magnesium deficiency in the rat described by Kruse, Orent and McCollum ('32) includes vasodilation and hyperemia of the vascular bed which is particularly evident in the ears, tail, and feet, hyperexcitability and convulsions a few days later, and, in those animals which survive, trophic changes of the epidermal structures and edema of the extremities.

The studies reported in this paper were stimulated by our interest in calcium requirements (Hegsted, Moscoso and Collazos, '52), the factors involved in adaptation to different levels of calcium intake (Hegsted, '52), and the effect of environmental temperature upon nutritional requirements (Hegsted and McPhee, '50). We noted that the vasodilation and trophic changes reported in magnesium dificiency were grossly similar to the changes observed in the ears, tails, and feet of animals kept at low temperatures. It seemed possible that magnesium might be involved. Recently, Platner and Hosko ('53) observed a rise in serum magnesium in various animals during hypothermia.

EXPERIMENTAL

The studies related to growth rates were done with weanling albino male rats obtained from the Charles River Breeding Laboratories. A total of 286 animals were used in 6 separate experiments. The number per group varied from 4 to 10 animals in the various experiments. The following variables were studied: magnesium level in the diet which

was fed at levels from 3 to 100 mg per 100 gm of diet, calcium content of the diet which was 200 mg, 600 mg or 1800 mg per 100 gm of diet, and environmental temperature which was either 55° ± 2°F. in the cold room or 78° ± 2°F. in the warm room. The animals received a purified diet of the following percentage composition: purified casein [2] 20, glucose 71.5, corn oil 5, cod liver oil 1, salt mixture 2.25, cystine 0.25, and choline chloride 0.3. Vitamins were added to supply 4 mg of thiamine hydrochloride, 8 mg of riboflavin, 4 mg of pyridoxine hydrochloride, 25 mg of calcium pantothenate and 40 mg of niacin per kilogram of diet. The salt mixture used was that of Jones and Foster ('42) with the $CaCO_3$ and $MgSO_4$ removed. When calcium and magnesium were added to the diet, these were in the form of $CaCO_3$ and MgO.

The animals were weighed twice or three times weekly, and each day at feeding time they were observed for lesions or signs of magnesium deficiency or cold injury.

RESULTS

Dietary calcium. As indicated by the review paper of Duckworth ('38–'39), there are considerable data indicating that the level of calcium intake influences magnesium metabolism and vice versa. Our data indicate an adverse effect of high calcium intakes, but this appears to be marked only at very low levels of magnesium intake. As shown in figure 1 at either zero or 3 mg of Mg/100 gm of diet, growth was better at a 200 mg level of calcium than at 600 or 1800 mg/100 gm. At the latter two levels of calcium, only one animal survived for a month at the zero level of magnesium, whereas three out of 4 animals survived at the low level of calcium. However, at levels of 6 and 12 mg of magnesium, there was no apparent effect of the calcium level. Hence we have been unable to arrive at any relationship, such as a calcium/magnesium ratio, that appears meaningful and consistent.

A summary of all the growth experiments is presented in table 1. Here again an inspection of comparable groups

[2] Shoffield.

fed 600 or 1800 mg calcium diets, particularly those in the cold, indicates that generally the animals fed the high calcium diet did less well than those upon the low calcium diet. This is especially evident in experiment V where all of the animals upon the 25 mg magnesium diet died within the first three weeks and the gain in weight at 50 or 100 mg of magnesium was considerably below that of those fed the same level of magnesium but the 600 mg level of calcium. We conclude that although high levels of calcium are somewhat detri-

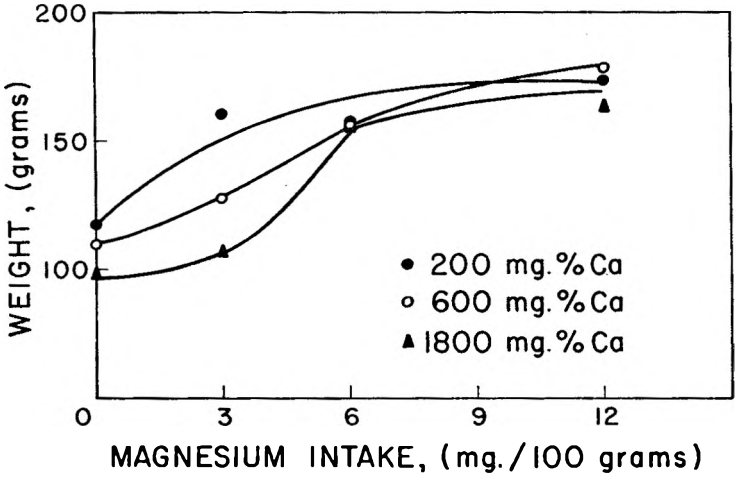

Fig. 1 The effect of low, medium and high calcium diets on the growth of rats fed various levels of magnesium (24-day period).

mental when the magnesium levels are critical, there are variables involved which were not under our control in these studies. A high calcium intake is detrimental at low levels of magnesium intake, but when the intake was near minimum requirements, the effect was too small to be detected in most of these studies.

In most of the experiments, maximum growth in the cold was obtained at about 50 mg of magnesium per 100 gm of diet regardless of whether the calcium intake was 600 or 1800 mg per 100 gm of diet.

Effects of low temperature. Although the temperature used in these studies may not be considered as particularly severe, it is sufficiently low to have a profound effect upon the growth of the animals. The cause of this growth inhibition is unknown. The growth studies are difficult to evaluate because

TABLE 1

Effect of magnesium and temperature on growth of rats

EXP.	ANIMALS PER GROUP	DIETARY Mg.	MEAN GAIN IN 24 DAYS			
			600 mg % Calcium		1800 mg % Calcium	
			78°F.	55°F.	78°F.	55°F.
		mg/100 gm	*gm*	*gm*	*gm*	*gm*
I	4	3	44		30 (1) [1]	
	4	6	64		67	
	4	12	84		77	
II	5	25	131	82 (2)	140	99 (2)
	5	50	133	98 (1)	145 (1)	116 (2)
	5	100	108	98	118	99 (1)
III	8	10		41 (2)		29
	8	20		64		53
	8	40		73		71
	8	80		80		70
IV	10	25		72 (1)		60
	10	50		83		74
	10	100		88		79
V	5	25	115	48	111	.. (5)
	5	50	118	80 (1)	114	58 (1)
	5	100	130	84	119	65 (2)
VI	6	25		.. (5)		
	6	50		80		
	6	100		81		

[1] Figures in parentheses indicate number of animals dead before 24th day.

the depressive effect of the low temperature is superimposed upon the dietary effect, and some animals apparently succumb from the temperature effects alone. Certain inherent differences in resistance to cold among the animals are clear but of unknown cause.

In attempting to evaluate the effects of the environmental temperature upon the magnesium requirements, we have

prepared figure 2 which is a weighted average, on the 24th day, of the gain in weight of all the animals from the groups shown in table 1. Since a consistent effect of the level of calcium in the diet was not apparent and since the number of animals at the two levels of calcium was essentially the same in either the warm or cold and at each level of magnesium studied, the effect of calcium has been ignored and

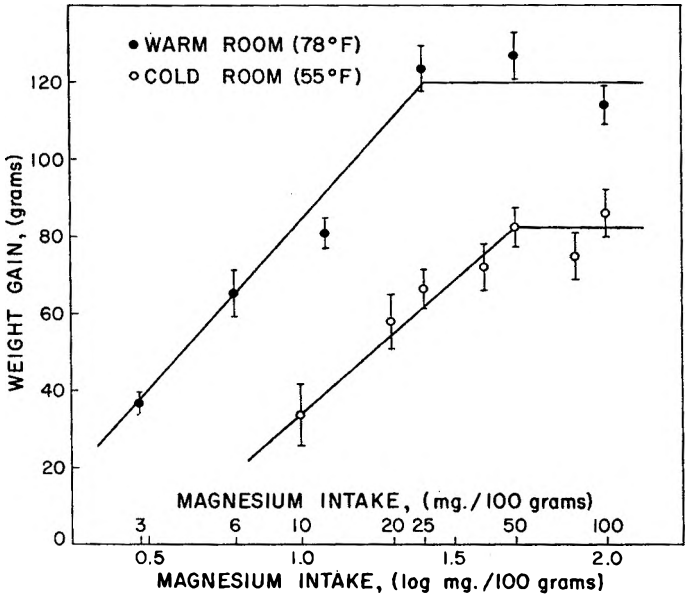

Fig. 2 The gains in weight of weanling rats fed various levels of magnesium during a 24-day period at two environmental temperatures. Vertical bars represent the standard error of the mean.

all the data at each level of magnesium have been averaged together. Regardless of whether this is justified or not, the results are sufficiently similar at each level of calcium so that the conclusion is not materially changed.

The fact that an essentially straight log-dose response curve is obtained in both the warm and cold rooms presumably means that magnesium was the chief determinant in the rate of growth obtained in either case. If such is the case, the magnesium requirement in the cold is markedly increased.

For example, to obtain a gain of 50 gm over the 24-day study period only 4 mg of magnesium per 100 gm of diet is required in the warm room, whereas the same gain is obtained at 16 mg of magnesium in the cold. The slopes of the lines may not be strictly parallel, but the magnesium need per unit diet to obtain comparable rates of growth appears to be from three to 5 times as great in the cold as in the warm environment.

If one compares the amount of magnesium required for maximum gain, these being different in the two temperatures, it appears that at the warm temperature the requirement is no more than 25 mg per 100 gm of diet. This is much higher than the previous estimate of 5 mg per 100 gm made by Tufts and Greenberg ('37–'38b). There is a suggestion in the data that levels as high as 100 mg may be slightly detrimental. On the other hand, the requirement in the cold is apparently at least 50 mg per 100 gm and perhaps somewhat higher in spite of the fact that the rate of growth and maximum weight obtained were considerably less than in the warm room.

Food consumption is obviously dependent upon the size of the animals, rate of growth and environmental temperature. In non-growing adult animals (see later) the food intake is about 20% higher in the cold room. In the growth experiments the cold-room animals usually were smaller at any given time and were growing less rapidly. The effect of the temperature on maintenance requirements may not have been comparable to the effect on the adults. Our data are insufficient to permit any careful comparisons of the calorie intakes, but it is clear that when actual magnesium intakes are considered, the difference in magnesium requirements in warm and cold environments is somewhat larger than indicated in figure 2.

As has been indicated, the early symptoms of magnesium deficiency and their sequels are similar in some respects to the effects of exposure to cold. These include a marked reddening of the ears, tails, and paws, but particularly evident upon the ears, presumably due to vasodilation and hyperemia.

These changes may be followed by a blanching with little apparent circulation and then edema and necrosis of the ears, tails, or paws. In our experience the latter changes are more characteristic of the effect of cold than of magnesium deficiency. In any event the similarity was sufficient to warrant study of the possibility that magnesium might be involved in the changes observed in animals in the cold room. It should be noted that frostbite is not involved since the cold-room temperature was only 55° ± 2°F.

Various attempts to establish a grading system failed because we are as yet unable to evaluate the relative importance of the various changes observed upon the outcome of the tissue. The degree of hyperemia or blanching at any particular time does not apparently bear any close correlation with the degree of necrosis or the amount of tissue which may eventually be lost. Also different groups of rats showed great variation in their susceptibility to cold injury. In some of the experiments, the groups of animals fed the high calcium diets suffered much more extensive injury than those upon the low calcium diets, sufficiently so that we felt confident of a dietary effect. Typical examples of animals on the high and low calcium diets (both receiving 100 mg % magnesium) after 14 days are shown in figure 3. In other experiments the effect could not be demonstrated, since little cold injury occurred. We have no evidence that the lesions are dependent upon the magnesium intake.

Balance studies. In an attempt to gain further information upon the effect of cold upon the metabolism of magnesium, a group of 12 adult female rats receiving the purified diet containing 50 mg of magnesium and 600 mg of calcium per 100 gm were placed upon a balance study. Food intakes were determined daily, and the urine and feces were collected at three- or 4-day intervals. During the first week the animals were in the warm room, and they were then placed in the cold room and collections continued for 4 additional weeks. The diet, urine and feces were analyzed for calcium and magnesium.

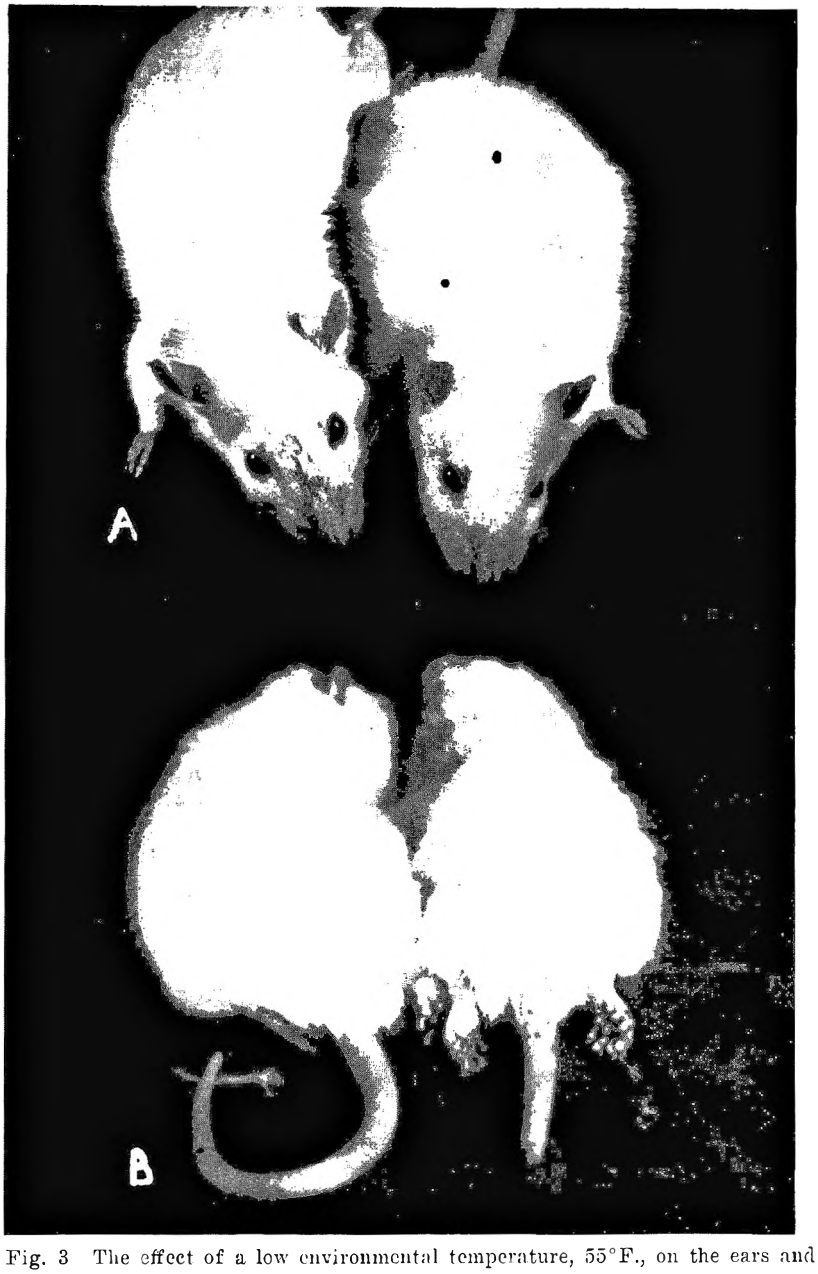

Fig. 3 The effect of a low environmental temperature, 55°F., on the ears and tails. Animal on the right fed the medium Ca diet (600 mg % Ca and 50 mg % Mg) showed less marked necrosis than the animal receiving the high calcium diet (1800 mg % Ca and 50 mg % Mg). The effects of diet and temperature were not consistent in different experiments (see text).

A summary of the data upon magnesium is shown in figure 4. The data show that magnesium balance is primarily related to the magnesium intake and secondly that the amount of magnesium required to maintain balance is somewhat greater in the cold room. While such data may at first hand seem to support the prior conclusion that the magnesium requirement is greater in the cold, we believe that this is not a necessary conclusion. Rather it is evident (Hegsted et al., '52; Hegsted, '55) that adult animals which maintain

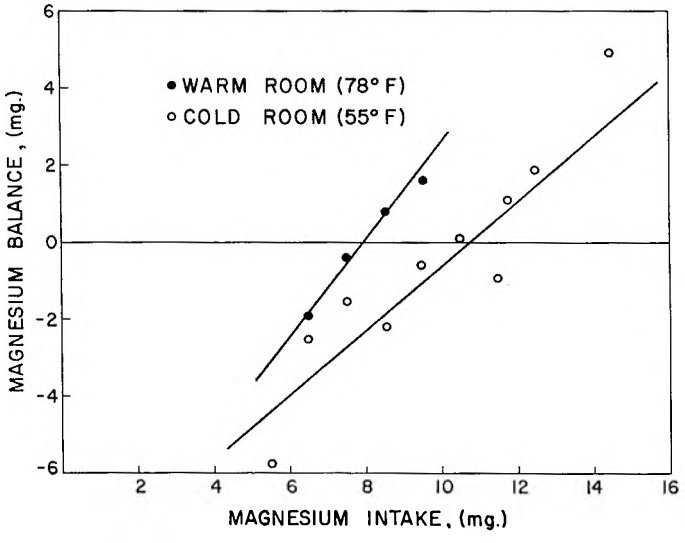

Fig. 4 The effect of environmental temperature on magnesium balance.

their weight must of necessity eventually come into balance at the level of intake they customarily consume. The food intake and thus the magnesium intake increases about 20% in the cold room. Hence they should come into balance at approximately 20% above the prior intake. This is essentially what the data show.

DISCUSSION

As has been indicated, an interpretation of the results does not appear possible at this time. Looking at the log-

dose response curve (fig. 2), it appears that the whole curve has been shifted to the right and lowered when the animals are placed in the cold. At each level of magnesium in the diet which can be compared, the growth in the cold is about 40 gm less than in the warm and this is also true at levels of magnesium which promote maximum gain at either temperature. At 50 mg % of magnesium, the animals in the warm room gained approximately 125 gm in 24 days (5 gm per day) while those in the cold gained slightly more than 80 (3 gm per day). Thus it is clear that the rates of growth and the efficiency of food utilization are considerably depressed in the cold. Actually, the efficiency of food utilization is about one-fourth as good as in the cold, since the rate of gain is about half, and the amount of food probably about 20% greater than in the warm. This is also about the same degree to which magnesium requirements are effected, about three to 5 times.

Magnesium may thus be in some way intimately related to the energy utilization. If it were only the rate of growth that was involved here, one might expect that the log-dose response curves would be essentially the same at low levels of magnesium and that only the maximum gain would be affected. If the magnesium requirement were only related to the amount of gain, then the cold-room animals would be expected to require the same or perhaps less in the diet in view of the slower rate of growth and the larger food intake.

In discussing the use of growth data for the evaluation of nutrition requirements, Hegsted ('48) has previously pointed out that an extension of the response curve to zero gain might be one method of estimating the maintenance requirement, provided the maintenance requirement per gram of diet were the same for the growing and adult animal. This concept has never been thoroughly explored. However, if it were applied to the data available from these experiments, the maintenance need is also some three to 5 times greater in the cold (depending upon whether the response curves are actually parallel or not). Assuming that they are parallel,

then they can be superimposed if the growth response is plotted in terms of the maintenance need at either temperature. (Since the curves are semilogarithmic, the situation does not improve if a standard correction of the maintenance need, perhaps 1 mg % in the warm and 4 mg % in the cold is made. This must be done in logarithms and is the same as plotting the values as percentage of the zero intercept.) Since practically all responses known in biology are proportional to the log of the dose (Bliss and Gyorgy, '51), it might be interesting to attempt comparison upon this basis when equivalence in response cannot be obtained, as when different species or different conditions are being compared. Magnesium deficiency would probably not be the nutrient of choice for such studies because of the high mortality at low doses. It would probably be difficult to determine the maintenance need (by the method proposed) because the lowest points on the response curve are influenced by this selection through death.

A great deal has been written upon stress, the general adaptation syndrome (Seyle, '46), etc., in recent years. Ershoff ('52) has reviewed much of the data related to the various nutritional factors. Ershoff and others have shown that animals deficient in various nutrients may be more susceptible to stress than normals. This may seem logical but the cause is in most instances unknown. We were unable (Hegsted and McPhee, '50) to demonstrate a difference in thiamine requirements due to temperature when account was made of the differences in food intake. The relationship which the present data upon magnesium may have to other work in the general field of stress and nutrition is only a matter of speculation at this time.

SUMMARY

Growth studies with weanling albino rats show that the magnesium required per gram of diet to promote equal gains in weight appears to be about 4 times higher at an environmental temperature of 55°F. than at 78°F. Since the maxi-

mum rate of gain is less at the lower temperature, the difference in the amount required for maximum gain is about twofold, 25 mg per 100 gm at 78°F. compared to 50 mg per 100 gm at 55°F.

High calcium diets were detrimental at low magnesium intakes. In some experiments high calcium diets apparently accentuate cold injury.

ADDENDUM

Recently Mudd and coworkers (S. H. Mudd, J. H. Park and F. Lipmann, Proc. Nat. Acad. Sci., *41:* 571, '55) demonstrated that the uncoupling of oxidative phosphorylation by iodothyronines *in vitro* could be prevented by the addition of magnesium. Thyroid stimulation may possibly be related to the increased magnesium requirement in the cold.

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EXPERIMENTS ON THE COMPARATIVE NUTRITIVE VALUE OF BUTTER AND VEGETABLE FATS

LESLIE P. DRYDEN, JOSEPH B. FOLEY,[1] PAUL F. GLEIS AND ARTHUR M. HARTMAN[2]

Dairy Husbandry Research Branch, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland

ONE FIGURE

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Apart from known differences in fat-soluble vitamin content, the nutritive value of butterfat as compared to vegetable fats has been a subject of dispute for a number of years (for reviews, see Cowgill, '45; Smith, '48; Deuel and Greenberg, '50). Workers in the field have frequently obtained conflicting results. In this paper there are considered a series of experiments which bear upon this problem, including some unpublished work conducted in this laboratory by Cary and associates (see footnote 1, table 1).

EXPERIMENTAL PROCEDURE

The experimental animals used were female albino rats primarily of the Wistar strain but derived from a colony maintained in this laboratory for many years. They were reared by colony mothers which were transferred at parturition from the stock ration to (experiments 1 to 3) ration no. 260 (see Dryden et al., '52, for composition) or to (experi-

[1] Present address: National Institutes of Health, Bethesda, Maryland.

[2] The earlier experiments described in this paper were carried out under the direction of Dr. Charles A. Cary, formerly Head, Division of Nutrition and Physiology, Bureau of Dairy Industry, retired.

ment 4) the sucrose-corn oil-sulfathalidine ration described below. The young were weaned at 25 days of age in experiments 1 to 3 and at 21 days of age in experiment 4. The weanling young were placed on the experimental rations as indicated and kept in individual cages provided with raised screen floors. All growth comparisons were made between litter mates, one rat from each litter being assigned to each diet in a particular experiment.

The composition of the 4.7% fat ration used in experiments 1, 2 and 3 was as follows: dextrin, 52.9%; lactose (U.S.P.), 14.6%; casein,[3] 23.4%; salt mixture,[4] 4.2%; butterfat,[5] or corn oil,[6] 4.7%; fish liver oil,[7] 0.02%; and vitamins,[8] 0.15%. For

[3] Crude commercial casein extracted with hot alcohol (Hartman et al., '51). In experiments 1 to 3, further extracted 16 hours with ether in a Soxhlet-type extractor.

[4] Salt mixture no. 12 (Jones and Foster, '42); modified in experiment 4 to contain the following additional ingredients (micrograms per gram of total salt mixture): $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 92; NaF, 506.

[5] Fed as whole unsalted butter that had been made with unpasteurized sweet cream by the Division of Dairy Products Research, Bureau of Dairy Industry, Beltsville, Md. Butter used in experiments 1 and 2 was made during July-October; that used in experiment 3 was made during March-May. After manufacture, the butter was stored at 0°F. or below until used.

[6] Mazola.

[7] Containing per gram, 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[8] Milligrams per 0.15 gm of vitamins (experiments 1 to 3), per 0.38 gm of vitamins (experiment 4), and per 100 gm of ration (experiments of Cary and associates), respectively: thiamine·HCl, 0.73, 1.60, 0.50; riboflavin, 0.91, 1.60, 1.00; pyridoxine HCl, 0.73, 1.60, 0.62; calcium pantothenate, 2.28, 10.00, 5.00; choline chloride, 109.31, 240.00, 250.00; nictotinic acid, 4.55, 10.00, 0.62; inositol, 9.11, 10.00, 100.00; para-aminobenzoic acid, 22.77, 60.00, 5.00; biotin, 0.009, 0.020, . . .; folic acid, 0.09, 0.20, . . .; ascorbic acid, 0.00, 10.00, 0.00; alpha-tocopherol acetate, 1.93, 20.00, 2.24; 2-methyl-1,4-naphthoquinone, 0.20, 0.50, 0.21; calciferol, 0.014, 0.020, 0.014; vitamin B_{12}, . . ., 0.010, . . .; synthetic vitamin A acetate (Myvax, Distillation Products Industries, Rochester, N. Y.), 0.00, 30000 I.U., 0.00; β-carotene, 0.00, 0.00, 0.56. In experiments 1 to 3, vitamin B_{12} was fed separately at the rate of 1 μg/rat/day. In the experiments of Cary and associates, daily supplements of biotin (0.5 μg), solubilized liver extract (50 mg) and APA liver extract (0.05 ml) were fed to each rat; the two latter supplements served as sources of folic acid and vitamin B_{12}. The folic acid was kindly supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and the crystalline vitamin B_{12} by Merck and Co., Inc. Rahway, N. J.

the 16.0, 30.7, and 40.0% fat rations, fat was substituted for dextrin isodynamically. Thus these rations provided on a calorie basis, equal intakes of protein, lactose, salts and added vitamins. It is evident, moreover, that the 40.0% fat ration contained no dextrin.

The basal ration used in experiment 4 had the following composition: sucrose, 47.6%; casein,[3] 20.0%; salt mixture,[4] 4.0%; butterfat,[9] margarine fat[9] or corn oil,[6] 28.0% and vitamins,[8] 0.38%. Sulfathalidine, when fed, was included in the rations at 2% in place of an equal amount of sucrose.

Fresh diets were made at approximately weekly intervals and stored in sealed Mason jars at 27°F. or below between feedings. Glass feeders were used and a complete change of food was made each week. The rations and distilled water were supplied ad libitum. Separate supplements, where given, were administered orally by syringe.

The procedure followed by Cary and associates (see table 1) was essentially the same as that described above, except that the experimental rats consisted of 21-day-old weanling stock colony young. The basal ration used by these workers had the following percentage composition: carbohydrate (lactose or dextrin), 33.4; casein,[10] 33.4; salt mixture (Jones and Foster, '42), 5.2; fat (butterfat or corn oil), 28.0; and added vitamins.[8]

RESULTS AND DISCUSSION

Comparison between butterfat and corn oil with lactose diets

With purified diets, Boutwell et al. ('43a) found that butterfat was superior to corn oil in promoting growth of weanling rats fed high fat diets only when lactose was the sole carbohydrate in the ration. In experiments carried out in this laboratory by Cary and associates (table 1), greater body weight gains were likewise observed on lactose rations containing

[9] Rendered from butter or oleomargarine purchased on the retail market. Equal quantities of 4 different brands were used.

[10] Two extractions of 6 hours each with hot 95% alcohol; then 16 hour extraction in Soxhlet with diethyl ether.

butterfat than on similar rations containing corn oil whereas such differences were not found on dextrin rations. In the Beltsville work, no significant differences in efficiency of food

TABLE 1

Body weight gains, efficiency of food utilization and body composition of rats fed purified diets containing butterfat or corn oil for a period of 8 weeks [1]

TYPE OF RATION		NO. OF RATS	AV. GAIN IN BODY WEIGHT	EFFICIENCY OF FOOD UTILIZATION [2]	BODY COMPOSITION [3]			
Carbohydrate	Fat				Moisture	Dry weight basis		
						Protein	Fat	Ash
			gm		%	%	%	%
Lactose	Butterfat	13 ♂	249	40	60.3	48.9	43.3	8.0
Lactose	Corn oil	13 ♂	229	39	61.4	50.0	40.0	8.3
Dextrin	Butterfat	13 ♂	284	41	56.2	42.6	52.2	6.8
Dextrin	Corn oil	13 ♂	289	42	57.6	45.2	49.2	6.7
F [4]			28.3**	3.7*				
Least signif. diff.			15.3	2.2				
Lactose	Butterfat	12 ♀	157	31	60.1	48.7	42.3	9.5
Lactose	Corn oil	12 ♀	136	28	62.5	52.2	36.8	10.6
Dextrin	Butterfat	12 ♀	168	32	57.1	43.5	47.8	8.2
Dextrin	Corn oil	12 ♀	164	31	58.4	45.2	47.9	7.9
F [4]			11.9**	2.7				
Least signif. diff.			12.0					

[1] Data from unpublished work carried out at Beltsville by C. A. Cary, E. W. J. Butz and M. S. Shorb, prior to Dr. Cary's retirement.

[2] Grams gained/grams food consumed × 100.

[3] Figures for body composition are based on a smaller group of rats: 7 male litters and 6 female litters. The values given represent the averages for two groups of 3 to 4 rats each. Each group was analyzed as a pooled lot. Since figures for individual animals are not available, these data have not been examined statistically.

[4] The symbol ** adjacent to or in conjunction with a *F* value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

utilization were observed between the two fats. In the work of Boutwell et al., differences in gross carcass analysis between the butterfat and corn oil groups on the lactose rations were not found to be sufficiently large to account for the superior weight gains of rats fed butterfat. At Beltsville,

with the female rats on the lactose rations, about one-third of the average difference in net live weight could be accounted for by an increased amount of fat laid down in the butter rats, about one-half by moisture and the remainder by ash and protein. With the male rats, however, the analysis indicated that most if not all of the average difference in net live weight observed was due to increased fat.

Barki et al. ('50) emphasized the importance of the level of fat at which comparisons of animal and vegetable fats have been made. In a comparison of several fats fed at three levels (10, 28 and 35%) in a casein-sucrose basal ration, these workers found greater weight gains for successively higher levels of butterfat but depressed growth with the highest level of corn oil. Boutwell et al. ('43b), using a skimmilk powder basal ration, likewise observed depressed growth with corn oil as the level of fat in the ration was increased from 25 to 35% of fat but found little change in growth with butterfat as the fat level was increased between these limits.

In table 2 are shown comparisons made in this laboratory between butterfat and corn oil fed in casein rations containing lactose. Tests were made at 4 different fat levels (4.7, 16.0, 30.7 and 40.0%). In agreement with the Wisconsin results (Barki et al., '50), greater weight gains were observed as the level of fat in the butterfat rations was increased. Although, at the higher levels of corn oil, weight gains were somewhat greater than at the lower levels, the differences were not significant. This latter result does not agree with the findings of Barki et al. ('50) or those of Boutwell et al. ('43b) in that no depression was found at the higher levels of corn oil. As between butterfat and corn oil, growth at the 4.7% level tended to be slightly greater on the average with corn oil. At the 16% level, there was little difference between weight gains on the two fats. At the higher levels, there was some indication of better growth on butterfat, the difference being statistically significant at the 40% fat level at the end of the 6th week in experiment 1 and approaching

statistical significance at a similar period in experiment 2 with the 30.7% fat ration. It was observed, however, that with 40% of corn oil, some separation of liquid fat gradually occurred and indeed it seemed impossible to prevent it completely in this ration at any level above about 31%. It is, of course, difficult to say just what effect such separation might

TABLE 2

Comparative rat growth obtained with diets containing corn oil or butterfat (with or without a linoleic acid supplement)

EXP.	FAT IN DIET	NO. OF LITTER-MATE RATS PER RATION	WEEKS ON EXP.	AVERAGE GAIN IN BODY WEIGHT		
				Butter [1]		Corn oil
				No supplement	Linoleate [2]	No supplement
	%			*gm*	*gm*	*gm*
1	4.7			103	...	112
	16.0	4	6	109	...	112
	40.0			126	...	116
1	4.7			164	...	169
	16.0	4	19	176	...	170
	40.0			194	...	186
2	4.7			119	120	124
	30.7	10	6	140	139	131

[1] Butter from cows on pasture used in experiment 1; butter from cows on barn feeds in experiment 2.

[2] Fifty milligrams/day. During the first 4 weeks of the experiment, ethyl linoleate was fed. This substance was prepared in this laboratory from corn oil according to the method of McCutcheon ('42). The iodine number of each preparation was determined by the Wijs method and found to be within 2% of the theoretical value. Samples more than three weeks old were not used. During the last two weeks of the experiment, pure methyl linoleate obtained from the Hormel Foundation, Austin, Minnesota, was fed.

have had on the relative weight gain but it will be observed that the magnitude of the average difference in weight gains in experiment 2 with the 30.7% fat rations, where no separation occurred, was similar to that obtained in experiment 1 with the 40% fat ration. The difference between butterfat and corn oil did not increase as the experimental period was extended beyond 6 weeks and at 19 weeks was not significant.

On the other hand, differences between growths obtained on the various fat levels did tend to increase in this later period.

Although the data are not given here for these experiments or for later experiments in this paper, increased food consumption was again found to accompany increased growth, just as had been observed in the experiments in table 1. Deuel and Movitt ('44), on the one hand, and Boutwell et al. ('44) and Nieman et al. ('52), on the other hand, among others, have discussed the relation between increased growth and food intake, the question of palatability, and other aspects of this problem. The presumption is made here that an increased food intake would be expected in most cases to accompany such increased weight gains as were observed in the present experiments.

Barki et al. ('50) found that supplementation of the 10% butterfat ration with ethyl linoleate failed to improve growth. From experiment 2, table 2, it can be seen that even a level of butterfat as low as 4.7% appeared to be adequate in essential fatty acids under these conditions.

Thus it is evident from these experiments that with purified diets containing lactose, as well as with skimmilk powder diets (Boutwell et al., '43b) and purified sucrose diets (Barki et al., '50), the level of fat in the ration is an important factor in demonstrating differences between butter and corn oil in promoting weight gains of young rats. Only at the higher fat levels was there evidence to indicate that butter promoted superior weight gains.

Comparisons of summer and winter butter

Boer, Jansen and their associates (Boer and Jansen, '42; Nieman et al., '52) found that butter made from milk produced by cows on pasture (summer butter) gave growth superior to that made from milk produced by cows on barn feeds (winter butter) and consequently used summer butter for their comparisons between butterfat and vegetable oils. However, it appeared from their tests that the differences

observed between summer and winter butter could be eliminated if a sufficiently high level of vitamin D were fed to the rats. Geyer et al. ('47) and Nath et al. ('48) obtained results indicating that a seasonal difference might exist in the nutritive value of the liquid fraction of butter. It seemed possible that differences might still be disclosed between summer and winter butter if the proper fat level and test conditions were used.

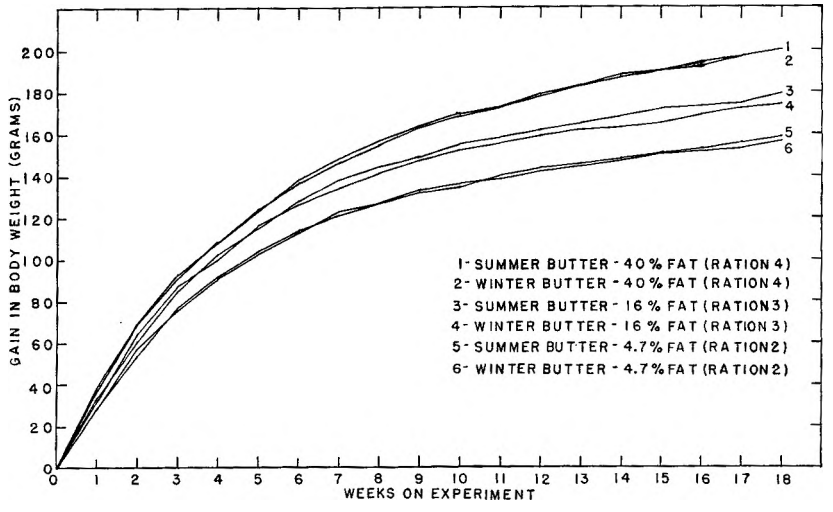

Fig. 1 Growth of weanling rats fed diets containing various levels of summer (pasture) butter and of winter (barn) butter over an 18 week period (experiment 3). Thirteen litters represented in each group. The butter was made from the milk of Holstein cows of the Beltsville herd. The cows used had been on either a barn or pasture ration for at least two months. The barn ration consisted of alfalfa hay, corn silage and a grain mix containing 13% dried beet pulp. The pasture ration consisted of blue grass or mixed orchard grass and ladino clover, grain and occasional hay supplements.

A direct comparison was made between the nutritive properties of butter made from milk collected from cows on barn feeds and those of butter made from milk collected from cows on pasture. The criterion used was the weight gains of weanling littermate young over an 18-week period. The basal ration was the same as the one used in the previous experiment. Comparisons were made at three different levels

of fat: 4.7, 16.0 and 40.0%. The results of this experiment are shown in figure 1.

When any one level of fat is considered, no significant differences are noted at any time during the experimental period between the average body weight gains of rats fed diets containing summer butter and those of rats fed diets containing winter butter. Thus the evidence indicates that under these experimental conditions, any unidentified growth-promoting factor which might be present in butter is contained in both summer and winter butter to a more or less equal degree.

The distinctly increased weight gains with the higher levels of butterfat are again evident. These differences were significant from the very first week of the experiment and continued to become somewhat more marked as the experiment progressed. After the 10th week, however, the differences in weight gains on the 4.7 and 16% fat diets remained constant, while the growth on the 40% fat diet continued to increase slightly relative to the other two levels till near the end of the experiment.

Comparisons involving rations containing sulfathalidine

Viswanatha et al. ('54) found that when sulfathalidine was included in a purified ration containing 28% of corn oil a depression in growth of weanling rats resulted which could be partially counteracted by replacing corn oil with butterfat. Thus under these conditions, they were able to demonstrate a differential effect upon growth in favor of butterfat.

In work in this laboratory, comparisons were made with a 28% fat basal ration similar to the sucrose ration used by Viswanatha et al. Three fats — corn oil, margarine fat and butterfat — were compared both with and without sulfathalidine in the ration. The rations were fed over an 8-week period.

The results at the end of the third and 8th weeks are shown in table 3. It can be seen that under these conditions, the

growth of the young receiving sulfathalidine was not depressed below that of the young not receiving this drug when butterfat or oleofat was fed. The inclusion of the drug in the ration fed to the mother and young prior to weaning may possibly account for this difference from the results of Viswanatha et al. with butterfat.

With the rations not containing sulfathalidine, no significant differences in growth were obtained between the three fats at any time during the experiment. With the rations

TABLE 3

Effect of sulfathalidine upon the post-weaning weight gains of rats fed corn oil, margarine fat or butterfat (exp. 4)

RATION [1]		AVERAGE GAIN IN BODY WEIGHT AT END OF	
Type	Fat	3 weeks	8 weeks
		gm	*gm*
Sulfathalidine	Corn oil	57	150
	Margarine fat	69	157
	Butterfat	75	158
No sulfathalidine	Corn oil	66	155
	Margarine fat	72	146
	Butterfat	69	153
F [2]		3.9**	1.0
Least significant difference		8.8	...

[1] Ten litter-mate rats on each ration.

[2] See footnote 4, table 1.

containing the sulfa drug, corn oil gave significantly inferior growth as compared to butterfat, in agreement with the results of Viswanatha et al., although the difference obtained here was not as great as they observed. Furthermore, all the differences were observed in the first three weeks of the experiment, the weekly weight gain on the corn oil ration begin as good as on butterfat after that time. To this extent, the results resembled those obtained in the experiments of Schantz et al. ('40), in which various vegetable oils and butterfat were compared when homogenized into liquid skim milk.

By the 8th week, the total growth on the corn oil ration, while still averaging less than that on the butterfat ration, was no longer significantly different. In this experiment, margarine fat was essentially as good as butterfat in promoting growth above that obtained with corn oil. In this rather high fat ration, corn oil was again found to gradually separate to some extent over a period of time. Presumably this does not account for the poorer growth on the corn oil-sulfa ration, however, since such separation likewise occurred on the corn oil-non-sulfa ration, where no growth differences were obtained.

The suggestion has been made that, in experiments of this sort, butterfat may supply an unidentified nutrient which is not present in corn oil but which may be supplied by intestinal synthesis in the absence of sulfathalidine. It is also possible that the effect of the fat is more indirect. Thus butterfat may counteract to some extent the effect of sulfathalidine in preventing the synthesis of such a nutrient. Boutwell et al. ('45) have shown an effect of the kind of dietary fat upon the requirements of the rat for certain known vitamins, presumably due to a varying effect of the different fats on the intestinal flora of the rat and consequently upon the intestinal synthesis of these substances.

SUMMARY

Experiments have been described dealing with the relative nutritive value of butterfat and vegetable fats. Comparisons at 4 different fat levels have been made between butterfat and corn oil incorporated into lactose-containing diets fed to weanling rats. Growth on the butter rations increased with increasing fat level. Growth on the corn oil rations showed the same tendency but not to a significant degree. Only at the higher fat levels was there evidence to indicate that butter promoted weight gains superior to those obtained with corn oil. Supplementation of the butter diet with linoleate had no effect.

A comparison between the growths of rats fed summer or winter butter at three different fat levels showed no differences at any level over an 18-week period.

Comparisons were made between butterfat, margarine fat and corn oil incorporated into sucrose rations containing 28% fat. Butterfat and margarine fat were found to promote significantly better growth of weanling rats than corn oil but only during the first three weeks of the experiment and when sulfathalidine was included in the ration.

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THE INFLUENCE OF ASCORBIC ACID AND THE SOURCE OF THE B VITAMINS ON THE UTILIZATION OF CAROTENE[1]

HELEN L. MAYFIELD AND RICHARD R. ROEHM
Department of Home Economics Research, Montana State College, Agricultural Experiment Station, Bozeman

ONE FIGURE

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Recent reports indicating that certain dietary constituents may influence the utilization of carotene have been summarized by Kemmerer ('52), Baumann ('53) and Quaife ('54). The work of Bassett, Loosli and Wilke ('48) indicated that an interrelationship exists between vitamin A and ascorbic acid in the nutrition of foxes and minks. Storvick, Hathaway and Nitchals ('51), working with human beings, found a statistically significant correlation between serum vitamin A and serum ascorbic acid. These studies suggest a possible relationship between body stores of vitamin A and ascorbic acid.

The study reported in this paper was planned to investigate the influence of the supplementary feeding of ascorbic acid on the conversion of carotene to vitamin A in the rat as measured by the storage of vitamin A in the livers and

[1] This study was a part of the Western Regional Cooperative Project W-4, Nutritional Status and Dietary Needs of Population Groups in Selected Areas of the West, Subproject 2, Biological Availability and Interrelationships of Nutrients in Foods. It was financed in part from funds appropriated under Research and Marketing Act of 1946. Contribution from Montana State College, Agricultural Experiment Station. Paper no. 365 Journal Series.

kidneys. Since it has been reported that the synthesis of ascorbic acid in certain animals may be dependent upon the levels of dietary thiamine (Sure, Theis and Harrison, '39), consideration was also given to the thiamine intake in the diet of the expermental rats.

EXPERIMENTAL PROCEDURE

Young rats [2] 21 days old and weighing 40 to 50 gm were assigned at random to one of 20 experimental groups. Each group consisted of 8 rats, 4 females and 4 males with the exception of two groups. The rats were placed in individual metal cages with raised screen bottoms and fed ad libitum the vitamin A-free test diet until they showed evidence of vitamin A deficiency as characterized by declining weight or ophthalmia, or both. The test diet consisted of vitamin A-free casein 18,[3] salt mixture 4,[4] brewers' yeast 8 [5] (0.1% of this irradiated), cornstarch 65, and cottonseed oil 5%.[6] In general, the Pharmacopoeia ('50) specifications for depletion and test diet used in the bio-assay of vitamin A were followed.

When the rats were depleted of vitamin A, one group serving as a control was killed and livers and kidneys were removed and analyzed for vitamin A in order to determine the degree of depletion. Four groups (1, 2, 3 and 4) were given supplementary feedings of 30 μg of carotene daily; 6 groups (5 through 10), 60 μg, and 9 groups (11 through 19), 120 μg of carotene for 14 days. In order to determine the effect of simultaneous feeding of ascorbic acid on the utilization of carotene, rats in these groups received daily either 0, 25, 50 or 75 mg of ascorbic acid.

[2] Obtained from Holtzman Rat Company, Madison, Wisconsin.

[3] "Vitamin Test" casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Salt Mixture, U.S.P. XIV, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Brewers' yeast as designated in text, yeast-a, Nutritional Biochemicals Corporation, Cleveland, Ohio; yeast-b, General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Wesson oil.

Certain groups (8, 9, 10, 14, 15 and 16) received the B vitamins from a test diet in which 5% of a synthetic vitamin mixture [7] was substituted for the 8% of brewers' yeast. The cornstarch was increased from 65 to 68%. This vitamin mixture contained no thiamine and these rats were fed daily supplements of 2.5 μg of thiamine hydrochloride in 0.1 ml of 95% ethyl alcohol. This low level of thiamine was fed since it has been shown that the amount of thiamine in the diet may affect the biological synthesis of ascorbic acid (Sure, et al., '39). Other groups received the B vitamins from either yeast-a or yeast-b [5] since with the development of the study it seemed advisable to determine if certain results would be repeated when another source of yeast was fed.

All rats in groups 1 through 19 received their supplements daily for 14 days. They were killed 24 hours after the last supplement was fed and pairs of kidneys and whole livers were removed, freed of foreign tissue, weighed, wrapped in aluminum foil, frozen and held at −10°F. until analyzed for vitamin A.

Supplements fed. The carotene, 90% beta-, 10% alpha-,[8] used in this study was recrystallized from benzene using methanol as the precipitant. It was dried in vacuum and then dissolved in cottonseed oil [6] with the aid of ether. The ether was subsequently removed by means of vacuum, and the carotene solution diluted with oil and supplemented with alpha-tocopherol so that 0.2 ml of the test oil contained either 30, 60 or 120 μg of carotene together with 0.50 mg of alpha-tocopherol. The concentration of the carotene in the test oils was checked periodically by colorimetric analyses. The as-

[7] The vitamin mixture was that suggested by Boutwell et al. ('45) with minor modifications based on more recent work; the addition of vitamin B_{12} was based on the report by Betheil and Lardy ('49): pyridoxine hydrochloride 0.63 mg, calcium pantothenate 5.0 mg, choline chloride 250.0 mg, niacin 0.63 mg, p-aminobenzoic acid 30.0 mg, inositol 100 mg, vitamin K 0.21 mg, biotin (free acid) 10.0 μg, folic acid 20.0 μg, riboflavin 0.40 mg, vitamin B_{12} 3.0 μg, and cornstarch to make 5 gm.

[8] Carotene, 90% beta 10% alpha, obtained from General Biochemicals Inc., Chagrin Falls, Ohio.

corbic acid supplements were prepared daily by dissolving the crystalline vitamin in distilled water so that 0.1 ml of solution contained 25 mg of ascorbic acid. The supplements were fed daily by pipetting the solutions directly into the mouths of the rats. Rats in group 4 were fed 50 mg of ascorbic acid daily by stomach tube to insure and check on the consumption of this factor. Test diets were fed ad libitum and the rats were weighed daily during the 14-day experimental period.

Vitamin A analyses. The method used for the vitamin analyses of the liver and kidneys was essentially that proposed by Kemmerer [9] and Vavich and Kemmerer ('50). Whole livers or pairs of kidneys were digested for 30 minutes on a steam bath in amounts of 12% alcoholic KOH proportional to the weights of the samples. This digest was extracted with purified ether, washed with water and extracted with three or more additional portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and diluted to appropriate volume. Aliquots of the ether solution were placed in Evelyn colorimeter tubes and evaporated on a hot water bath with the aid of reduced pressure. The residue was taken up at once in dry chloroform. Then the tube was placed in an Evelyn photoelectric colorimeter, the $SbCl_3$ reagent was added rapidly and the maximum displacement at 620 mμ was read. A standard curve was prepared using crystalline vitamin A acetate [10] in dry chloroform. The amount of vitamin A present in the whole liver and pairs of kidneys was calculated for each rat, and the mean, together with the standard error of the mean, was determined for males and females. When possible the resulting data were treated statistically according to the analysis of variance (Ostle, '54).

[9] Personal communication, Dr. A. R. Kemmerer, University of Arizona.

[10] Crystalline vitamin A acetate, Nutritional Biochemicals Corporation, Cleveland, Ohio.

RESULTS

Data showing the effects of feeding various levels of ascorbic acid to rats receiving 30, 60 or 120 mg of carotene together with the B vitamins from yeast-a, yeast-b or a syn-

TABLE 1

Mean weight and mean vitamin A content of livers and kidneys from rats fed (1) 30 or 60 μg of carotene daily, (2) the B vitamins from different sources and (3) various levels of ascorbic acid

GROUP NO.[1]	ASCORBIC ACID FED	SEX	GAIN IN BODY WEIGHT	WEIGHT OF		VITAMIN A IN	
				Liver	Kidneys	Liver	Liver plus kidneys
	mg/day		*gm*	*gm*	*gm*	*μg*	*μg*
				30 μg carotene per day yeast-a[2] source of B vitamins			
1	0	F	42	5.6	1.1	39.5	46.5 ± 4.5[3]
		M	66	7.5	1.3	37.9	52.0 ± 10.4
2	25	F	39	5.0	1.1	60.0	64.9 ± 4.2
		M	69	7.7	1.2	24.4	38.0 ± 4.6
3	50	F	47	6.2	1.2	38.1	46.2 ± 6.0
		M	65	7.1	1.3	18.3	34.6 ± 2.7
4	50[4]	F	38	5.6	1.0	42.2	46.1 ± 3.1
		M	55	6.5	1.2	22.5	36.7 ± 2.9
				60 μg carotene per day yeast-b[5] source of B vitamins			
5	0	F	48	6.1	1.2	88.3	90.8 ± 10.7
		M	79	8.4	1.4	92.3	102.3 ± 6.7
6	25	F	39	6.1	1.2	106.7	111.0 ± 10.6
		M	81	9.2	1.5	68.5	76.1 ± 5.4
7	50	F	43	5.9	1.2	74.4	79.8 ± 7.2
		M	66	8.0	1.5	62.2	74.4 ± 16.2
				60 μg carotene per day synthetic vitamin mixture[6] source of B vitamins			
8	0	F	44	5.9	1.2	119.3	121.6 ± 13.7
		M	65	7.1	1.3	103.4	113.4 ± 12.0
9	25	F	53	7.0	1.3	141.3	144.3 ± 10.9
		M	67	8.0	1.4	98.6	106.5 ± 2.7
10	50	F	52	6.9	1.3	114.4	117.3 ± 7.1
		M	66	7.0	1.4	90.2	103.0 ± 4.1

[1] Eight rats per group, 4 females (F), 4 males (M).
[2] Yeast-a, brewers' Yeast U.S.P., Nutritional Biochemicals Corportation, Cleveland, Ohio.
[3] Standard error of the mean
[4] Fed by stomach tube.
[5] Yeast-b, brewers Yeast, General Biochemicals, Inc., Chagrin Falls, Ohio.
[6] See text, p. 205, for synthetic vitamin mixture.

thetic vitamin mixture are summarized in tables 1 and 2. Each group, except 18 and 19, consisted of 8 rats, 4 females and 4 males. Group 18 had 7 rats, 4 females and 3 males, and group 19, 6 rats, 3 females and 3 males. Since preliminary examination of the data showed that the males and females did not always respond in the same manner to the supple-

TABLE 2

Mean weight and mean vitamin A content of livers and kidneys from rats fed (1) 120 μg of carotene daily, (2) the B vitamins from different sources and (3) various levels of ascorbic acid

GROUP NO.[1]	ASCORBIC ACID FED	SEX	GAIN IN BODY WEIGHT	WEIGHT OF		VITAMIN A IN	
				Liver	Kidneys	Liver	Liver plus kidneys
	mg/day		*gm*	*gm*	*gm*	*μg*	*μg*
				yeast-b [2] source of B vitamins			
11	0	F	54	6.3	1.2	158.4	161.2 ± 14.9 [3]
		M	81	8.8	1.4	122.5	131.8 ± 18.4
12	25	F	38	5.7	1.2	173.0	175.1 ± 19.6
		M	76	8.4	1.6	125.8	134.1 ± 7.2
13	50	F	51	6.1	1.3	182.4	185.3 ± 21.4
		M	83	8.7	1.4	149.9	154.7 ± 6.1
				synthetic vitamin mixture [4] source of B vitamins			
14	0	F	45	6.2	1.1	198.8	201.1 ± 25.7
		M	64	7.1	1.2	173.8	178.9 ± 10.1
15	25	F	51	6.1	1.2	197.7	200.0 ± 12.2
		M	67	7.3	1.4	176.8	182.0 ± 8.1
16	50	F	47	5.6	1.1	222.9	225.0 ± 23.7
		M	75	8.0	1.5	189.7	195.5 ± 32.1
				yeast-a [5] source of B vitamins			
17	0	F	40	6.1	1.1	191.9	194.2 ± 27.0
		M	63	7.7	1.2	179.4	185.7 ± 16.4
18 [6]	50	F	39	5.4	1.1	222.6	225.5 ± 21.0
		M	76	7.7	1.3	196.9	202.7 ± 2.5
19 [7]	75	F	48	5.6	1.1	207.5	209.9 ± 24.4
		M	74	8.8	1.5	169.1	182.0 ± 0.6

[1] Eight rats per group, except as noted — 4 females (F), 4 males (M).
[2] Yeast-b, brewers' Yeast, General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Standard error of the mean.
[4] See text, p. 205, for synthetic vitamin mixture.
[5] Yeast-a, brewers' Yeast U.S.P., Nutritional Biochemicals Corporation, Cleveland, Ohio.
[6] Seven rats in group, 4 females, 3 males.
[7] Six rats in group, 3 females, 3 males.

mentary feeding of ascorbic acid when the storage of vitamin A in the liver and kidneys is used as the criterion, the data are presented separately for the two sexes.

All rats were depleted of vitamin A in from 21 to 23 days. Vitamin A analyses of the livers and kidneys of the group of rats serving as a control and killed at the end of depletion indicated that there was no measurable amount of vitamin A left in these organs. At the end of the depletion period, the mean weight was 102 gm for females and 104 gm for males. However, at the end of the 14-day test period, the male rats weighed considerably more than did the females and the mean weights of whole livers and kidneys from the males were somewhat greater than those from the females (tables 1 and 2).

In all groups, with the exception of group 5, the amount of vitamin A found in the whole livers of the females was larger than that found in the males. In a reverse manner, the males in each group had more vitamin A in their kidneys than did the females. This relationship of vitamin A in the livers and kidneys of male and female rats has been noted by many workers and discussed in detail by Booth ('52) and Arnrich and Morgan ('54). All comparisons and discussions that follow are based on the total amount of stored vitamin A (livers plus kidney).

Female rats — effect of ascorbic acid on vitamin A storage

Female rats fed 25 mg of ascorbic acid daily and either 30 or 60 μg of carotene (table 1, groups 2, 6 and 9) stored considerably more vitamin A in their livers plus kidneys than did similar females receiving no ascorbic acid (groups 1, 5 and 8). However, when the female rats received 50 mg of ascorbic acid (groups 3, 4, 7 and 10) the amount of vitamin A stored in the liver and kidneys dropped to a level similar to or below that found when no ascorbic acid was fed. This higher level of ascorbic acid, 50 mg, appeared to have a de-

pressing effect on the conversion of carotene to vitamin A and its subsequent storage in the liver and kidneys of the female rats receiving 30 or 60 mg of carotene. These findings are graphically presented in figure 1, section 1.

Female rats fed 120 μg of carotene and either 0 or 25 mg of ascorbic acid (table 2 and fig. 1, section 2) showed in one

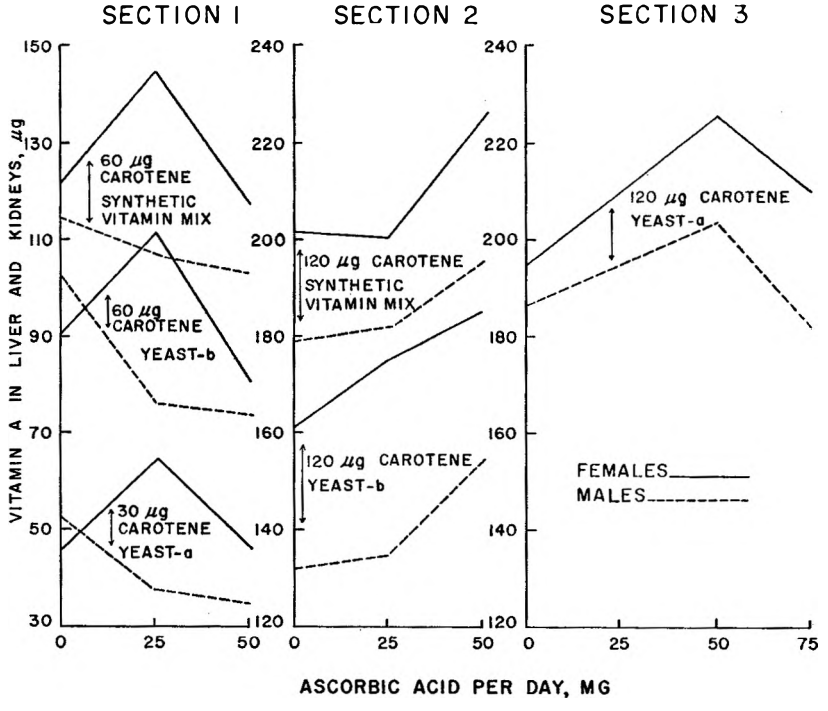

Fig. 1 A graphic presentation of the amount of vitamin A in the livers and kidneys of rats fed (1) 30, 60 or 120 μg carotene daily, (2) the B vitamins from different sources and (3) various levels of ascorbic acid.

case no response to 25 mg of ascorbic acid and slight response in the other. When 50 mg of ascorbic acid were fed considerably more vitamin A was found in the livers and kidneys than at the 0 or 25 mg level. Since 50 mg of ascorbic acid appeared to have a reverse or depressing effect when fed to rats receiving either 30 or 60 μg of carotene, it seemed desirable to investigate the effect of an even higher level

of ascorbic acid on rats receiving 120 μg of carotene. Groups 17, 18 and 19 receiving 120 μg of carotene were fed 0, 50 and 75 mg of ascorbic acid. Results given in table 2 and shown graphically in figure 1, section 3, indicate that the females in group 18 fed 50 mg of ascorbic acid stored more vitamin A than did those in group 17 receiving no ascorbic acid. However, feeding 75 mg of ascorbic acid to rats receiving 120 μg of carotene had a depressing effect on the utilization of carotene similar to that obtained in groups 3, 7 and 10.

Male rats — effect of ascorbic acid on vitamin A storage

Male rats receiving 25 or 50 mg of ascorbic acid daily utilized less effectively the supplementary feedings of either 30 or 60 μg of carotene than did those receiving no ascorbic acid (table 1). The graphic presentation of these results, shown in figure 1, section 1, indicates that at these levels of carotene intake the male and female rats responded to the feeding of ascorbic acid in an opposite manner. Less than 25 mg of ascorbic acid was not fed so it is not known if a smaller amount of this factor would have favorably influenced the utilization of carotene by the male rats.

Feeding 25 mg of ascorbic acid to male rats receiving 120 μg of carotene did not affect the utilization of carotene as indicated by liver and kidney storage (table 2 and fig. 1, section 2). However, when fed 50 mg of ascorbic acid, the male rats stored more vitamin A than did those receiving no ascorbic acid. A comparison of groups 17, 18 and 19 indicates that the male rats in group 18 on a 50 mg level of ascorbic acid responded in a similar manner. Feeding 75 mg of ascorbic acid (group 19) had a depressing effect on liver storage of vitamin A in the male rats as it did in the female rats.

Yeast versus a synthetic vitamin mixture

Rats in groups 8, 9 and 10, and groups 14, 15 and 16 received the B vitamins with the exception of thiamine from a

synthetic vitamin mixture which contained the known vitamins in amounts believed to be necessary and adequate for the normal nutrition of the rat. The daily supplement of thiamine hydrochloride, 2.5 μg per rat, was an amount previously determined in this laboratory as sub-optimum for thiamine nutrition but one on which rats would subsist. As indicated by the weights of rats at the end of the test, this low level of thiamine did not significantly affect the gains in weight of the rats during the two-week test period.

The rats, both males and females, receiving the diet with the synthetic vitamin mixture utilized more effectively the 60 and 120 μg levels of carotene than did those receiving the diet with the yeast-b which was fed during the same experimental period. This was true at all three levels of ascorbic acid intake, 0, 25 and 50 mg. An analysis of variance shows that this difference is highly significant with an F value of 33.44; an F value of 7.01 is necessary for significance at the 1% level.

This diet containing a synthetic vitamin mixture with its low thiamine supplement was studied in comparison with the diet containing yeast because of indications that the level of thiamine in the diet might influence the synthesis of ascorbic acid by the rat (Sure, et al., '39). It was thought that these rats might respond differently to the supplementary feeding of ascorbic acid than did those receiving a generous supply of thiamine as in the diet containing yeast. While the rats receiving the diet with a synthetic vitamin mixture utilized the carotene more effectively than did those on the diets containing yeast, it will be noted that they responded to the supplementary feeding of ascorbic acid in a similar manner (fig. 1). Hence, it appears that some factor other than the low level of thiamine is responsible for the greater utilization of the carotene. It may be that either (1) the yeast contained some factor which retarded the utilization of carotene, or (2) the synthetic vitamin supplement contained an amount of some factor or a ratio of various factors that brought about greater utilization of the carotene.

Using the vitamin analyses of yeast-b furnished by the producer,[11] a comparison of the two diets shows the following approximate relationships: (1) niacin 4.6 times greater in yeast diet, (2) folic acid 20 times greater in yeast diet, (3) riboflavin about the same in both diets, and (4) choline 8.8 times greater in the synthetic vitamin mixture diet. Since High and Wilson ('53) suggest an interrelationship between vitamin B_{12} and the metabolism of carotene, samples of yeast-b were analyzed for vitamin B_{12} content[12] in order to make a comparison of this factor in the two diets. Using the *O. malhamensis* assay figure of 1.0 mμg per gm of yeast-b, the vitamin B_{12} content of the synthetic vitamin mixture diet was 375 times greater than that of the yeast-b diet. These comparisons of the two diets, together with the work of High and Wilson ('53), suggest that the vitamin B_{12} content of the diet containing a synthetic vitamin mixture might be the factor responsible for the greater utilization of the carotene. Further tests are being made to determine if vitamin B_{12} is the influencing factor.

Yeast-a was fed to groups 1, 2, 3, 4, 17, 18 and 19 and yeast-b to groups 5, 6, 7, 11, 12 and 13. The resulting data suggest that these two yeasts may differ in their influence on the utilization of carotene (fig. 1).

Statistical treatment of data

The data obtained in groups 5 through 16 were treated according to the analysis of variance.[13] The F values obtained indicate that the variation in the utilization of carotene between rats fed the two diets and between males and females were highly significant (F values of 33.44 and 18.13 respectively; F value necessary for significance at the 1%

[11] See footnote 5.

[12] Vitamin B_{12} analyses of yeast-b made by the Wisconsin Alumni Research Foundation: *Ochromonas malhamensis* assay — about 1 m μg per gm. *Lactobacillus leichmannii* assay — 0.80 m μg per gm yeast.

[13] Grateful acknowledgement is made to Dr. Bernard Ostle, Statistical Laboratory, Montana State College for supervising these analyses.

level, 7.01). The F value for the variations among levels of ascorbic acid indicated that they were not significant. However, further analysis of the data indicated a significant interaction between levels of ascorbic acid and carotene with an F value of 4.19. (F value of 3.13 necessary for significance at the 5% level; 4.92 necessary at the 1% level.) These F values, together with the presentation of the data in figure 1, indicate that this relationship of ascorbic acid and carotene is significant. As previously discussed, ascorbic acid under certain circumstances increases the utilization of carotene, while under others it has no effect or may even decrease its utilization.

DISCUSSION

Other workers have reported varying effects of certain compounds on the conversion of carotene to vitamin A. Johnson and Baumann ('48) and Swick and Baumann ('52) have reported that feeding high levels of certain tocopherols to rats receiving carotene lowered the amount of vitamin A stored in the livers and kidneys. Hebert and Morgan ('53) found that a daily supplement of 0.5 mg of alpha-tocopherol to rats receiving 87 to 174 μg of carotene daily produced an increase in liver storage of vitamin A.

High and Day ('51) found that small amounts of lutein fed to rats receiving different levels of carotene increased the quantity of vitamin A stored in the livers and kidneys, but large amounts decreased the storage. High, Woods and Wilson ('52) and High, Smith, Taylor and Wilson ('54) report similar effects when certain antioxidants were fed to rats receiving carotene. High et al. ('54) state that their study affords further evidence that the mode of action of the antioxidants studied is concerned with their antioxidant activity, and that in large amounts they may suppress the oxidative processes which are probably involved in the enzymatic conversion of carotene to vitamin A.

Since ascorbic acid has antioxidant properties, these reports support the findings of this study that small levels of

ascorbic acid may increase the utilization of carotene while larger amounts may have no effect or may decrease its utilization. As Hebert and Morgan ('53) found with alpha-tocopherol, this study indicates that these amounts of ascorbic acid vary with the level of carotene that is fed.

The greater liver and kidney storage of vitamin A in female rats as compared to male rats has been well recognized (Booth, '52). However, other workers have not reported an inverse response by males and females to the compounds studied such as was found in this experiment. When 30 or 60 μg of carotene were fed, the male rats responded in an opposite manner than did the females to the feeding of ascorbic acid.

SUMMARY

Male and female rats receiving 30 or 60 μg of carotene did not respond in the same manner to the supplementary feeding of 25 or 50 mg of ascorbic acid. When fed 120 μg of carotene and either 25, 50 or 75 mg of ascorbic acid, the response in the two sexes was similar.

Female rats fed 25 mg of ascorbic acid and either 30 or 60 μg of carotene stored more vitamin A in their livers and kidneys than did those receiving no ascorbic acid. When fed 50 mg of ascorbic acid, vitamin A storage dropped to a level similar to or below that found when no ascorbic acid was fed.

Male rats fed 25 or 50 mg of ascorbic acid and either 30 or 60 μg of carotene stored less vitamin A in their livers and kidneys than did those receiving no ascorbic acid.

Male or female rats fed 25 mg of ascorbic acid and 120 μg of carotene showed, with one exception, no increase in liver storage of viamin A. Vitamin A storage was increased when the rats were fed 50 mg of ascorbic acid, but storage was decreased when they were given 75 mg.

Male and female rats fed the diet containing the synthetic vitamin mixture, used as a source of the B vitamins, utilized more effectively the 60 and 120 μg levels of carotene than did those receiving the diet containing yeast. This was true at the three levels of ascorbic acid intake.

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THE QUANTITATIVE AMINO ACID REQUIREMENTS OF YOUNG WOMEN

III. TRYPTOPHAN [1]

RUTH M. LEVERTON,[2] NORMA JOHNSON,[3] JEAN PAZUR AND JOAN ELLISON

Nebraska Agricultural Experiment Station, University of Nebraska, Lincoln, Nebraska

ONE FIGURE

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The necessity for supplying tryptophan preformed in the diet to maintain human subjects in nitrogen equilibrium has been reported by Holt et al. ('41), Rose ('49), and Rose et al. ('54). Holt and co-workers ('44), found that following a period of tryptophan deficiency nitrogen equilibrium could be restored in two men subjects when the intake reached 6.0 to 9.0 mg of L-tryptophan/kg/day. Baldwin and Berg ('49) kept 15 men in equilibrium for 12 days or more on a total daily intake of 225 mg of L-tryptophan. Rose, Lambert and Coon ('54) have studied the tryptophan requirement of three men and found that 300 mg daily was sufficient for them as well as for 36 men who were subjects for studies of amino acids other than tryptophan.

Tryptophan was the third amino acid included in the study of the amino acid requirements of young women which was conducted at the University of Nebraska.[4] The purpose of the

[1] Published with the approval of the Director as Paper no. 707 Journal Series, Nebraska Agricultural Experiment Station.

[2] Present address: Division of Home Economics, Oklahoma A. and M. College, Stillwater.

[3] Present address: Spokane, Washington.

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work was to find the least amounts of threonine, valine, tryptophan, phenylalanine and leucine which would maintain the subjects in nitrogen equilibrium under the controlled conditions of the study.

PROCEDURE

In the report on threonine Leverton et al. ('56a) have described in detail the plan of the study, the description of the subjects, and the nitrogen balance technique. Also given were the composition of the basal ration of cornstarch, sugar, butter fat, corn oil, agar flakes, lemon juice, and mineral and vitamin supplements.

Two groups of subjects were used for the study of tryptophan requirement. Group A included 8 college girls who are identified here as subjects 31, 32, 34, 35, 36, 37, 39 and 41. Each subject was studied on several different levels of tryptophan intake. Group C included another 8 college girls, identified as subjects A through H. They were used to check the adequacy of the lowest level of tryptophan which had been found to maintain nitrogen equilibrium in all of the subjects in group A. This checking was done simultaneously for all 5 of the acids which were studied, tryptophan, threonine, valine, phenylalanine, and leucine. Each of these acids was included in the ration, referred to as the Test Mix, in the smallest amount which the initial study had indicated would meet the needs of every subject already studied. The composition of the amino acid mixture for group A, based on the amounts in 20 gm of egg protein, and of the Test Mix for group C are shown in table 1.

The nitrogen intake for both group A and group C was 9.5 gm daily, except for subject 31. Because this subject was slow coming into nitrogen equilibrium, her intake was increased to 12 gm of nitrogen and the amino acids were included in amounts equivalent to 27 gm of egg protein. Glycine was the chief source of nitrogen for group A, and glycine and diammonium citrate supplied equal portions of the nitrogen for the members of group C.

A few auxiliary foods were used to make the diet more acceptable. Subjects in group A and group C received daily: 100 gm reconstituted frozen orange juice, 75 gm canned pineapple, and 75 gm canned peaches. Also group A had 25 gm raw carrots, and group C had 25 gm grape juice. These were assayed microbiologically with *Leuconostoc mesenteroides* and the results checked with *Streptococcus faecalis*. The tryp-

TABLE 1

The purified amino acids fed in the study of tryptophan requirement

AMINO ACID	GROUP A	GROUP C
	Amount equivalent to 20 gm egg protein [1]	Amount in Test Mix
	gm/person/day	*gm/person/day*
L-Arginine·HCl	1.549	1.549
L-Histidine·HCl	0.519	0.519
L-Lysine·HCl	1.800	1.800
L-Tyrosine	0.900	0.900
L-Phenylalanine	1.260	0.200
L-Tryptophan	0.300 [2]	0.150
L-Cystine	0.480	0.480
L-Methionine	0.820	0.820
L-Threonine	0.980	0.184 [3]
L-Leucine	1.840	0.600
DL-Isoleucine	3.200	3.200
L-Valine	1.460	0.550
Nitrogen content	1.910	1.583

[1] Subject 31 received an amount equivalent to 27 gm of egg protein.

[2] The amount of tryptophan was varied during the study of requirement.

[3] Subjects C and F required 275 mg plus 30 mg from the auxiliary foods.

tophan content of the foods used daily ranged from 6 to 7 mg. In reporting the results 7 mg is added to the intake of purified L-tryptophan even though it is a small proportion of the total intake. All of the intakes described throughout the text refer to the L-isomer of tryptophan.

Aliquots of individual foods and chemicals were analyzed for nitrogen before being fed and the daily nitrogen intakes of all of the subjects did not vary more than 0.2 gm throughout the study.

RESULTS

The mean daily nitrogen balance of each subject studied on each level of tryptophan intake is charted in figure 1. Connecting the mean values for all subjects on each intake with the solid line emphasizes the downward course of the nitrogen balances with decreasing tryptophan intakes. The shaded

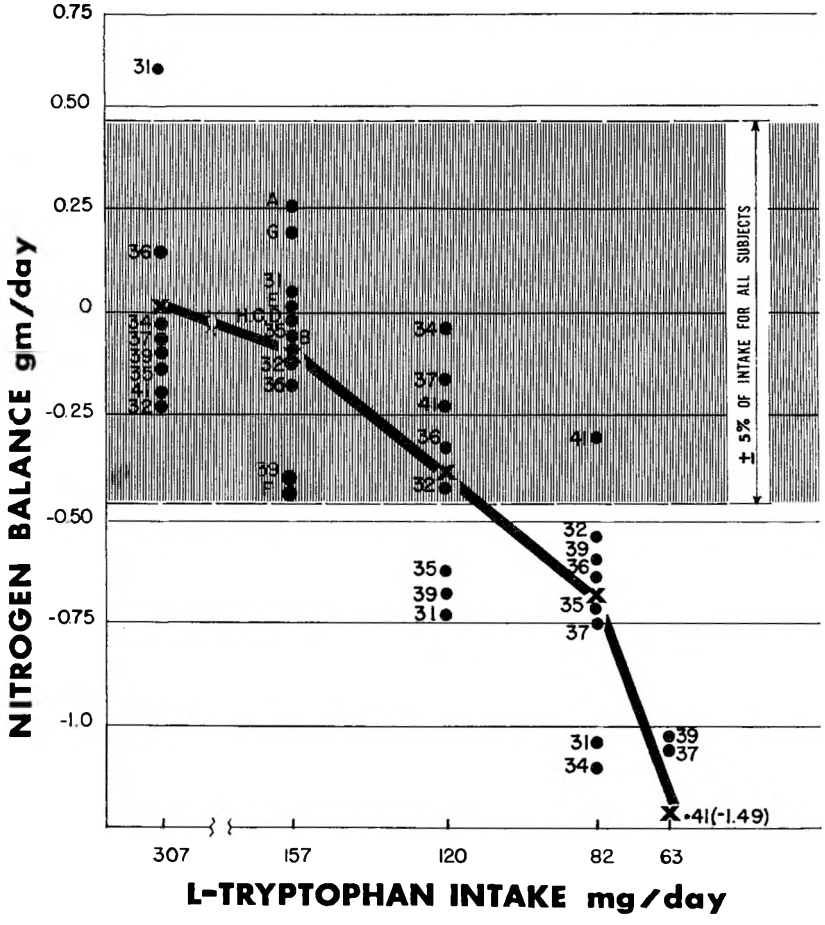

Fig. 1 Tryptophan intake and nitrogen balance. Sixteen subjects for 285 subject-days. Each subject is identified by her code which appears beside her retention for each level on which she was studied.

X = mean retention of all subjects on each level of intake.

Intake includes 7 mg/day supplied by auxiliary foods.

portion of figure 1 denotes the zone of equilibrium or the area where the difference between the nitrogen intake and excretion, called the balance, is within ± 5% of the nitrogen intake. Not until the excretion is more than 105% of the intake is the balance considered to be definitely negative.

The individual data have been summarized in table 2 to show the mean daily nitrogen balance, standard deviation,

TABLE 2

Nitrogen balance of subjects on different levels of tryptophan intake

LEVEL OF L-TRYPTOPHAN [1]	NUMBER OF SUBJECTS ON EACH LEVEL	TOTAL NUMBER OF SUBJECT-DAYS	NITROGEN BALANCE				NUMBER OF SUBJECTS IN NEGATIVE BALANCE [3]
			Mean	S.D.[2]	Range		
					Low	High	
mg/day			*gm/day*		*gm/day*	*gm/day*	
Group A							
307 [4]	8	56	0.01	0.26	— 0.21	0.59	0
157	5	38	— 0.13	0.16	— 0.39	0.05	0
120	8	73	— 0.39	0.26	— 0.73	— 0.04	3
82	8	56	— 0.70	0.26	— 1.10	— 0.30	7
63	3	11	— 1.19	0.26	— 1.49	— 1.02	3
Group C Test Mix							
157	8	52	0.00	0.19	— 0.40	0.25	0
Groups A and C							
157	13	90	— 0.05	0.19	— 0.40	0.25	0

[1] Includes 7 mg supplied by the auxiliary foods.

[2] Standard deviation.

[3] Excretion more than 105% of the intake.

[4] One subject, number 31, received 406 mg.

and range for each group of subjects, together with the total of the number of days the subjects were studied and the number of subjects in negative balance on each intake of tryptophan.

The 8 subjects in group A were in nitrogen equilibrium or storing nitrogen during 7 days on the first level of intake of 307 mg of tryptophan daily. The intake was then reduced to 82 mg of tryptophan daily for 7 days and 7 of the subjects, all except subject 41, went into negative balance. Because subject

41 was still in equilibrium on 82 mg, and because the negative nitrogen balances had not been large for some of the other subjects, it was decided to reduce the intake of subjects 37, 39, and 41 to 63 mg of tryptophan daily. They immediately went into marked negative balance. For the three days subject 37 was on this intake, her mean daily nitrogen balance was —1.06 gm, and corresponding figures for subject 39 for 4 days, and subject 41 for three days were —1.02 gm and —1.49 gm respectively. The intake of these three subjects was then increased to 120 mg tryptophan daily which was the amount being given to the other 5 subjects.

TABLE 3

Test of significance of differences between nitrogen balances

LEVELS OF L-TRYPTOPHAN INTAKE	MEAN DIFFERENCE	N	DEGREES OF FREEDOM	t
mg/day	*gm N/day*			
63 vs 82	0.49	11	9	2.722 [1]
82 vs 120	0.31	16	14	2.385 [1]
120 vs 157	0.26	21	19	3.487 [2]
157 vs 307	0.14	21	19	0.615

[1] Significant at the 5% level of probability.
[2] Significant at the 1% level of probability.

A daily intake of 120 mg of tryptophan failed to maintain nitrogen equilibrium in three of the 8 subjects in group A, but when their intake was increased to 157 mg of tryptophan daily, they promptly came into nitrogen equilibrium.

The results secured with group A led to the decision to include 150 mg of purified L-tryptophan in the Test Mix for group C in addition to the 7 mg supplied by the auxiliary foods. The total of 157 mg daily was sufficient to keep the 8 subjects A through H in nitrogen equilibrium. Although these subjects were on only the one level of tryptophan intake, 157 mg, the balances of subjects B, C, D, E and H were similar to those of subjects 31 and 35 on this intake, and subjects 31 and 35 were in negative balance on the next lower intake of 120 mg.

TABLE 4

Lowest intakes of tryptophan used in this study which maintained nitrogen equilibrium [1]

DESCRIPTION	SUBJECT CODE	L-TRYPTOPHAN INTAKE		NO. DAYS	MEAN NITROGEN BALANCE	MEAN CREATININE COEFFICIENT	MEAN CALORIC INTAKE	AGE
		mg/day	*mg/kg$^{3/4}$/day*		*gm/day*		*cal/kg/day*	*yr.*
Group A								
Subjects who	31	157	7.9	7	0.05	25.4	41.6	26
were studied on	35	157	6.6	8	— 0.05	18.2	35.8	20
both higher and	39	157	7.2	7	— 0.38	19.3	40.4	20
lower intakes	32	120	4.7	5	— 0.40	20.7	35.5	22
than shown here	34	120	5.2	7	0.34	21.2	38.2	21
	36	120	5.4	7	— 0.32	19.9	39.4	23
	37	120	5.4	10	— 0.15	19.9	41.4	19
	41	82	4.0	6	— 0.30	21.2	41.1	23
Group C Test Mix								
Subjects who	A	157	6.9	4	0.25	23.2	37.9	20
were studied	B	157	7.4	7	— 0.06	21.6	42.7	22
only on an intake	C	157	6.7	7	0.00	21.6	36.8	19
of 157 mg	D	157	7.7	7	0.00	20.0	45.4	19
	E	157	7.5	7	0.02	22.7	45.2	20
	F	157	7.7	4	— 0.40	22.0	44.7	22
	G	157	7.0	7	0.20	22.9	41.3	22
	H	157	8.8	9	0.00	20.7	53.4	20

[1] Nitrogen excretion within 95 to 105% of the intake.

When the results are combined for group A and group C on an intake of 157 mg of tryptophan daily, the mean daily nitrogen balance for the 15 subjects is -0.05 ± 0.19 gm.

The "t" test for significance of differences between the nitrogen balances on the different levels of tryptophan intake is shown in table 3. The differences were significant except between the balances on 157 and 307 mg of tryptophan.

The smallest amount of tryptophan fed in this study which maintained nitrogen equilibrium in each subject in group A is shown in table 4, together with data on the creatinine coefficient, caloric intake, and age of each subject. Figures are also given for group C on the Test Mix. To consider differences which might be due to differences in the size of the subjects, the tryptophan intake is expressed as mg/kg$^{3/4}$ actual weight/day as well as the total daily amount. No relationships were observed between the amount of tryptophan needed and creatinine coefficient, the caloric intake required for maintaining body weight, or the age of the subjects.

The three subjects, 37, 39, and 41, who received only 63 mg of tryptophan daily for three or 4 days experienced considerable nausea. Subject 39 lost a meal, subject 37 had diarrhea, and subject 41 complained of extreme fatigue. During the 7 days she was on 120 mg of tryptophan subject 36 lost two meals. She was not nauseated but attributed the loss of meals to a muscle spasm. None of the subjects knew their level of tryptophan intake, when it was changed, nor that there might be any symptoms associated with an inadequate intake.

DISCUSSION

The amount of tryptophan needed for nitrogen equilibrium by the different subjects in this study varied from 82 to 157 mg per day. When calculated in terms of body weight the requirement of the subjects who were on several different levels of intake (group A) ranged from a low of 4.0 mg/kg$^{3/4}$/day for subject 41 to a high of 7.9 mg/kg$^{3/4}$/day for subject 31. The range also included the intakes of 7 of the 8 subjects in group C who were on only one level of intake, 157 mg.

All 13 of the women who were studied on a daily intake of 157 mg of tryptophan were in nitrogen equilibrium. On the basis of mean metabolic size, this was an intake of 7.3 mg/kg$^{3/4}$/day. Five subjects maintained equilibrium on 120 mg of tryptophan or a mean of 5.1 mg/kg$^{3/4}$/day. The results for the 8 subjects on the Test Mix demonstrate the adequacy of 157 mg of tryptophan daily when 4 other essential amino acids, threonine, valine, phenylalanine, and leucine, were present in the smallest amounts which had been found to be adequate for all of the subjects studied previously.

On the basis of the results reported here, it seems likely that 157 mg of tryptophan daily, which should be rounded to 160 mg, will meet the requirements of young women similar to the subjects in this study and under similar conditions.

The figure of 160 mg of tryptophan is similar to the figures given in the literature for the daily tryptophan requirement of men. Baldwin and Berg ('49) could not produce a distinctly negative nitrogen balance on a diet of mixed foods which supplied as little as 115 mg of tryptophan daily, and on a synthetic diet 15 subjects were kept in nitrogen balance with an intake of 225 mg of tryptophan. Rose, Lambert and Coon ('54) found 150 mg of tryptophan was sufficient for two young men but a third subject required 250 mg for nitrogen balance. They also report that 36 young men who were subjects for studies of other amino acids were in positive balance on 300 mg of tryptophan or less. Holt et al. ('44) found that intakes of 6 to 9 mg of tryptophan per kilogram, or 420 to 630 mg per 70 kg, would restore nitrogen equilibrium in men following tryptophan deficiency. They considered that these subjects maintained a good appetite during the two to 5 weeks they were on a diet without tryptophan.

Again, as in the case of threonine and valine (Leverton et al., '56a, '56b), it seems likely that ordinary foods can supply an adequate intake of tryptophan for people with needs similar to those of the subjects in this study. The patterns of amino acids consumed by humans in different parts of the world, as calculated by Block and reported by Allison ('53),

vary in tryptophan content from 3.2 to 7.0 gm per 100 gm of protein. Futrell et al. ('52) analyzed the self-chosen diets of 4 women and found daily intakes from 0.50 to 1.28 gm of tryptophan. Diets of common foods used by Wharton et al. ('53) in studies of nitrogen balance supplied 0.4 to 0.5 gm of tryptophan daily.

SUMMARY AND CONCLUSIONS

The tryptophan requirement has been studied using 8 young women as subjects. On a semi-purified diet nitrogen balances were determined on daily intakes which began with 307 mg of L-tryptophan, were reduced to 82 mg and then to 63 mg, and finally raised to 157 mg.

The lowest daily intake of tryptophan which maintained all of these subjects in nitrogen balance (excretion within 95 to 105% of the intake), was 157 mg. This level of tryptophan was fed to 8 additional subjects in a ration which contained minimum amounts of threonine, valine, phenylalanine, and leucine. These subjects remained in nitrogen balance.

The figure of 157 mg of L-tryptophan is rounded to 160 mg and suggested as a tentative minimum daily requirement for young women similar to those studied here.

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ROLE OF VITAMIN B_{12} IN NUCLEIC ACID METABOLISM

I. HEMOGLOBIN AND LIVER NUCLEIC ACID LEVELS IN THE RAT [1]

W. T. WONG [2] AND B. S. SCHWEIGERT

Division of Biochemistry and Nutrition, American Meat Institute Foundation and Department of Biochemistry, University of Chicago, Chicago, Illinois

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A decrease in the basophilic elements (RNA) was reported in the livers of vitamin B_{12}-deficient rats (Stern et al., '49; Seigel and Worley, '51). This observation was supported by quantitative studies from this laboratory. Both desoxyribonucleic acid (DNA) and ribonucleic acid (RNA), expressed either as the amount per unit of fresh weight, dry weight, or nitrogen content of liver, were significantly lower in livers of deficient animals than in the controls (Rose and Schweigert, '52; Schweigert et al., '54). This observation has been confirmed in the present study, employing an essentially similar ration containing the same amount (0.06%) of iodinated casein. In addition, liver regeneration studies after partial hepatectomy were also performed.

Due to the limitations in assessing the data obtained when iodinated casein was present in the ration, it was of importance to produce an uncomplicated vitamin B_{12} deficiency in rats without the use of the thyroid stimulant (iodinated casein) and to repeat our observations on the liver nucleic acid

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[2] The material presented has been taken from a thesis submitted to the Department of Biochemistry of the Division of Biological Sciences, University of Chicago, in partial fulfillment of the requirements for a degree of Master of Science, 1955.

content of these animals. This was accomplished by breeding mature female rats that were depleted of the vitamin and using the deficient young for biochemical and nutritional studies. With the dietary regimens selected from preliminary experiments, two extensive experiments were conducted in which the growth rates, liver DNA, and liver RNA were determined when no vitamin B_{12}, graded levels of vitamin B_{12}, and adequate vitamin B_{12} plus liver were fed. With deficient animals, a lower growth rate and a significantly lower content of liver DNA and RNA were observed. In addition the hemoglobin levels and the incidence of "porphyrin whiskers" and scaly feet were determined for vitamin B_{12}-deficient and supplemented animals.

EXPERIMENTAL

Animals. The rats used in this study were of the Sprague-Dawley strain, and were raised from vitamin B_{12}-deficient mothers with the exception of those used in experiment 1. In the latter case, male weanling rats were purchased. All animals, unless stated otherwise, were kept in individual cages in an air-conditioned room and fed and watered ad libitum.

High mortality of the young was observed in the preliminary reproduction and lactation experiments. This was probably due primarily to the dietary treatment, since this group of females was fed the vitamin B_{12}-deficient ration for two weeks before breeding. Other workers (Halvorson and Schultze, '50; Dryden et al., '52) have also observed a high mortality in newborn rats obtained from mothers fed similar diets. In view of these observations, subsequent tests were conducted with females fed a commercial stock diet [3] prior to breeding in addition to the use of improved management procedures during the lactation period. With these procedures a satisfactory level of deficiency was achieved and approximately 70% of the young given the mothers to raise were weaned.

[3] Rockland pellets.

Mature females fed a stock diet were placed in groups of 5 to 7 animals in large breeding cages, and were fed the basal ration which contained no iodinated casein. Male rats which had been fed the stock diet were rotated among the breeding cages every week.

After pregnancy was ascertained the females were transferred to individual cages. After parturition, the litters were reduced to 8, and the same vitamin B_{12}-deficient diet was fed throughout gestation and lactation. The young were weaned at 21 days of age. The adult females were then fed the stock diet for at least two weeks before being returned to the breeding cages. The basal ration was then fed as outlined above during the production of second litters of deficient young.

Rations. The composition of the basal diet is similar to that of the corn-soybean oil meal ration previously described (Scheid et al., '50). The salt mixture of Spitzer and Phillips ('46) was used at a 2.16% level. This ration was designated to be adequate in all known nutrients except vitamin B_{12}. Appropriate quantities of the basal ration and basal ration supplemented with graded levels of vitamin B_{12} and dried liver [4] were made up at regular intervals and refrigerated when not in use. Six-hundredths per cent of iodinated casein [5] was added to all diets in experiment 1.

Partial hepatectomy. At the end of the experimental period (experiment 1) the rats fed the 4 iodinated diets which contained, respectively, 0, 2, 50, and 50 μg of vitamin B_{12} per kilogram of ration plus 4% of dried liver, were subjected to an additional stress of partial hepatectomy. The excised portion of the liver was blotted and weighed. It was then cut up into small pieces and kept in a Petri dish in the deep freeze for nucleic acid analysis. Four days after partial hepatectomy the surviving animals were killed by decapitation, and the remaining liver excised, blotted and weighed. The scar tissues were then removed; the liver cut up with scissors, and stored in Petri dishes in the deep freeze prior to analysis.

[4] Wilson Laboratories.

[5] Protomone.

Determinations of DNA and RNA. For the colorimetric analysis of DNA [6] and RNA,[7] known standards were run concomitantly with samples. The nucleic acid contents of the standards were determined by acid hydrolysis and subsequent phosphorus analysis according to the method of Fiske and Subbarow ('25). DNA was determined by the diphenylamine method of Dische ('30), and RNA by the orcinol method (Mejbaum, '39). Both diphenylamine [8] and orcinol [9] were purified prior to use: Diphenylamine was recrystallized twice from Skellysolve B, and orcinol from benzene-Skellysolve B. Liver samples (after defrosting) were prepared for nucleic acid extraction and analysis by a modification of the method of Schneider ('45).

Nuclear count. In experiment I, 0.5 ml of the 10% liver homogenate was withdrawn and added to 19.5 ml of methyl green reagent (50 mg of methyl green in 100 ml of 0.9% saline containing 3% of glacial acetic acid), and the number of nuclei was counted with a hemocytometer. Nuclei counting was later abandoned since consistent results were difficult to obtain under the conditions of our experiments.

Hemoglobin. Blood was collected immediately after decapitation and shaken with the anticoagulant of Heller and Paul (Hawk et al., '54). Two-hundredths milliliter of blood was then added to 10 ml of 0.4% ammonia solution, and optical density was read with a Junior Coleman Spectrophotometer at 540 mμ. Hemoglobin concentration was obtained by comparing the optical density with that of a known standard.

RESULTS AND DISCUSSION

In tables 1 and 2 are summarized the results of experiment I. In this experiment 41 male weanling rats were fed the basal ration containing 0.06% of iodinated casein for two weeks. They were then distributed at random into 4 groups to which

[6] Courtesy of Dr. Mancourt Downing.

[7] Nutritional Biochemicals Corp., Cleveland, Ohio.

[8] Eastman.

[9] Eastman, anhydrous.

the iodinated casein basal diet supplemented respectively with 0, 2, 50, and 50 μg of vitamin B_{12}/kg + 4% dried liver were fed. The latter group was included to evaluate the possible need for any unidentified factor. After 4 weeks on experiment the rats were subjected to an additional stress of partial hepatectomy, and the liver tissue removed during and 4 days after partial hepatectomy was analyzed for DNA and RNA.

TABLE 1

Growth rates and liver nucleic acid composition of male rats fed iodinated casein rations (experiment I)

	RATION			
	Basal	Basal + 2 μg vit. B_{12} per kg	Basal + 50 μg vit. B_{12} per kg	Basal + 50 μg vit. B_{12} per kg + 4% liver
No. of rats	11	10	10	10
Body wt., *gm* [1]				
At weaning	48 ± 1 [2]	47 ± 1 [2]	46 ± 1 [2]	46 ± 1 [2]
At 6 wk.	212 ± 7	232 ± 7	246 ± 6	261 ± 4
Wt. gain, *gm* during 4 wk. exptl. period [1]	95 ± 6	113 ± 6	126 ± 6	141 ± 4
Hepatectomy				
% Liver removed [3]	54	60	63	62
Survival [4]	4/9	6/8	5/7	5/7
RNA, *mg/gm liver*				
Before hepatectomy	5.0 ± 0.19	5.7 ± 0.10	5.5 ± 0.25	5.5 ± 0.12
After hepatectomy	5.7 ± 0.29	5.4 ± 0.21	5.9 ± 0.17	6.7 ± 0.17
DNA, *mg/gm liver*				
Before hepatectomy	1.98 ± 0.056	2.25 ± 0.063	2.55 ± 0.092	2.35 ± 0.037
After hepatectomy	2.01 ± 0.042	2.19 ± 0.047	2.54 ± 0.057	2.51 ± 0.074

[1] Weanling rats were fed the vitamin B_{12}-deficient ration for two weeks and then randomized among all experimental groups and fed the indicated rations for 4 weeks before being subjected to partial hepatectomy.

[2] Mean and standard error of the mean.

[3] Total liver weight was calculated from liver/body ratios obtained by Schweigert et al. ('54), and in the present work. The following ratios were used = 0.067 for Basal, 0.059 for Basal + 2 μg B_{12}, and 0.051 for Basal + 50 μg B_{12}, with or without liver added.

[4] Number of animals survived 4 days after operation/number operated on. The difference in the latter number and total number in each group represents those rats that died during anesthesia.

It can be seen from table 1 that the DNA and RNA per gram of liver (fresh weight) are greater in the supplemented than in the deficient groups. The supplemented rats also gained weight more rapidly than the deficient ones, and the post-operative mortality appeared lower.

From the knowledge of the ratios of liver weight to body weight as observed previously (Schweigert et al., '54) and in the present work, the weight of the intact liver before partial hepatectomy was calculated. The difference between this calculated weight of the whole liver and the hepatectomized weight represented the amount left in the body of the operated animal (zero time). Four days later, the animal was killed and the weight of the liver ascertained. From these data, and from analysis for the DNA, RNA, and nuclei per gram of liver both before and after partial hepatectomy, the extent of regeneration could be readily calculated. These results are given in table 2.

It is interesting to note that the percentage regeneration of DNA approximated the regeneration for the liver weight. The percentage of RNA regeneration was higher than that for DNA for all groups except that fed the sub-minimum level of vitamin B_{12} (2 μg/kg).

On an over-all basis this experiment confirmed and extended previous findings from this laboratory (Rose and Schweigert, '52; Schweigert et al., '54). It seemed desirable to modify subsequent experiments to avoid the use of the iodinated casein by producing weanling rats for the experiments from vitamin B_{12}-deficient mothers. It was hoped that this technique would provide greater biochemical and nutritional differences associated with the deficiency, and greater uniformity of vitamin B_{12} reserves in the experimental animals since littermate comparisons could then be made with the dietary treatments chosen.

Table 3 summarizes the results of experiments II and III, in which the young obtained from these deficient mothers were fed, respectively, with non-iodinated casein and iodinated

TABLE 2

Liver regeneration of rats after partial hepatectomy. (Iodinated casein rations)

RATION	BASAL				BASAL + 2 μG VIT. B_{12}/KG				BASAL + 50 μG VIT. B_{12}/KG				BASAL + 50 μG VIT. B_{12}/KG + 4% LIVER			
	Before	Zero time	4 Days	% Regen.	Before	Zero time	4 Days	% Regen.	Before	Zero time	4 Days	% Regen.	Before	Zero time	4 Days	% Regen.
Wt., *gm* [1]	13.9	6.4	9.4	40	13.4	5.4	10.5	64	12.2	4.5	10.2	74	13.1	5.0	11.2	77
RNA, *mg* [1]	70	32	53	55	76	31	56	56	67	25	60	83	72	27	75	107
DNA, *mg* [1]	27.5	12.7	18.9	42	29.9	12.2	23.0	61	30.8	11.5	25.9	75	30.8	11.8	28.1	86
Nuclei [1] × 10^{-6}	1000	460	670	21	952	383	535	28	708	261	480	49	1140	435	785	50

[1] All amounts are expressed as average value per liver. For calculations of total liver weight, see footnote 2, table 1.

TABLE 3

Growth rates, ratios of liver weight to body weight, and liver nucleic acid composition of rats born from vitamin B_{12}-deficient mothers (experiments II and III)

RATION [1]	NO. OF ANIMALS [2]	SEX	WT. AT WEANING	BODY WT. AT 6 WKS.	LIVER WT. / BODY WT.	LIVER DNA	LIVER RNA
			gm	*gm*		*mg/gm*	*mg/gm*
A1	7/7	M	37 ± 2.3 [3]	166 ± 18 [3]	0.056	1.88 ± 0.048 [3]	5.65 ± 0.11 [3]
A2	4/5	M	39 ± 1.4	187 ± 11	0.052	1.80 ± 0.049	6.11 ± 0.10
A3	6/6	M	37 ± 2.8	231 ± 8	0.044	2.10 ± 0.025	6.73 ± 0.18
A4	5/5	M	35 ± 1.9	236 ± 5	0.044	2.04 ± 0.070	6.54 ± 0.17
A1	4/5	F	34 ± 1.0	116 ± 5	0.052	1.99 ± 0.082	5.87 ± 0.28
A2	4/4	F	34 ± 2.7	153 ± 9	0.047	2.15 ± 0.040	6.43 ± 0.21
A3	5/5	F	32 ± 1.9	169 ± 5	0.041	2.54 ± 0.130	6.66 ± 0.14
A4	5/5	F	34 ± 2.7	171 ± 4	0.040	2.58 ± 0.150	6.67 ± 0.17
B1	1/5	M	35	92	0.077	1.92	6.70
B3	4/4	M	33	183	0.053	2.73	9.03
B4	2/2	M	33	209	0.051	2.56	8.50
B1	1/2	F	38	78	0.067	1.96	6.85
B3	1/1	F	38	166	0.055	3.08	8.31
B4	2/2	F	28	170	0.055	2.84	8.36

[1] For detailed description of the basal ration see text. Rations designated A contain no iodinated casein. Those designated B contain 0.06% iodinated casein; no. 1 = deficient diet, no. 2 = 2 μg of vit. B_{12}/kg supplement, no. 3 = 50 μg vit. B_{12}/kg supplement, and no. 4 = 50 μg vit. B_{12} + 4% dried liver supplement.

[2] Number alive at end of experiment/number started.

[3] Mean and standard error of the mean.

casein diets for a period of 6 weeks after weaning. Five out of 7 animals fed the iodinated casein basal diet could not withstand the stress of the dietary regime and died, as contrasted with 100% survival of animals fed the iodinated-casein diets supplemented with vitamin B_{12}. An excellent growth response was observed when adequate levels of vitamin B_{12} were included in the ration. Thus the use of vitamin B_{12}-deficient mothers was shown to be an excellent technique for producing deficient young, and might be suitably modified to become an animal assay for vitamin B_{12}. An additional advantage is that the vitamin B_{12} deficiency thus produced was not complicated by the presence of iodinated casein in the rations. As observed in experiment I (table 1), the groups fed the iodinated casein diet containing adequate vitamin B_{12} and liver supplement tended to show a slight increase in growth rate as compared with those fed the diet containing 50 μg of vitamin B_{12} alone. The general trend of higher ratios of liver weight to body weight was also apparent for the deficient animals; the ratios of the comparable group fed iodinated casein being higher than the other. The liver DNA and RNA values were low for the deficient groups, which is in accord with results of the first experiment. It is also of interest that the females showed the deficiency more readily than the males, as judged by the nucleic acid content of the liver. The inclusions of iodinated casein in the diet aggravated the deficiency symptoms, whether judged by mortality, growth rate, liver DNA or liver RNA content.

During the course of this investigation, it was observed that young rats obtained from deficient mothers showed a syndrome of porphyrin whiskers and scaly feet (especially the hind paws). These effects became apparent after the animals were fed the deficient diet for two to three weeks. The degree of severity of the scaly feet syndrome was observed to be 4 to 10 times greater with the rats fed rations containing 0 or 2 μg of vitamin B_{12} per kilogram. A greater incidence of porphyrin whiskers among the deficient groups was also observed.

Table 4 summarizes the results obtained in a subsequent experiment. An additional diet containing adequate vitamin B_{12} plus 10% of liver was also included. In addition to DNA, RNA, and other measurements, blood hemoglobin level and liver nitrogen were also determined.

The general trend of a decrease in liver nucleic acid, but essentially no change in liver nitrogen in the deficient animals,

TABLE 4

Growth rates, ratios of liver weight to body weight, and blood hemoglobin levels, of rats born from vitamin B_{12}-deficient mothers and fed graded levels of vitamin B_{12} with or without liver (experiment IV)

RATION [1]	NO. OF ANIMALS	SEX	WT. AT WEANING	BODY WT. AT 6 WKS.	LIVER WT. / BODY WT.	HEMOGLOBIN
			gm	*gm*		%
A1	7	M	41 ± 1 [2]	154 ± 13 [2]	0.053	14.8 [3]
A2	7	M	40 ± 2	175 ± 10	0.050	14.4
A3	8	M	41 ± 2	257 ± 6	0.045	15.3
A4	6	M	41 ± 3	261 ± 6	0.047	15.6
A5	5	M	40 ± 2	260 ± 9	0.048	15.8 [4]
A1	5	F	38 ± 2	125 ± 9	0.047	15.2 [5,7]
A2	4	F	38 ± 3	128 ± 4	0.052	15.1
A3	3	F	38 ± 1	181 ± 8	0.042	15.2
A4	5	F	39 ± 1	179 ± 5	0.041	16.4 [6]
A5	4	F	42 ± 1	182 ± 6	0.042	16.2 [6]

[1] See footnote 1 of table 3. A5 = 50 μg vit. B_{12}/kg + 10% dried liver supplements.

[2] Mean and standard error of the mean.

[3,4,5,6] Differences between 3 and 4, and 5 and 6, are statistically significant ($P < 0.05$).

[7] Average of determinations on 4 animals.

was again observed. These data supported and confirmed the observations of experiment II. The inclusion of dried liver in the ration did not improve growth over and above that due to vitamin B_{12}. However, the presence of liver in the diet appeared to be important in promoting hematopoiesis, as evidenced by an increase of hemoglobin in the blood. This increase becomes significant ($P < 0.05$) in male rats fed the ration containing 10% of liver and in female rats fed the

rations containing either 4 or 10% of liver. Detailed histological and cytochemical studies now in progress [10] may reveal further differences attributable to vitamin B_{12} or liver supplementation.

Vitamin B_{12} has not as yet been assigned a definite metabolic function. It was reported to play a role in citrovorum factor formation (Doctor et al., '53), the latter being implicated in nucleic acid synthesis (Jukes, '53). Conceivably, the vitamin might act on another important pathway leading to nucleic acid synthesis. It is known that ribose and desoxyribose can be formed by liver enzyme systems by condensation of C_2 and C_3 compounds (Racker, '52; McGeown and Malpress, '52, '54). If a lower concentration of these compounds to form the C_5 sugars was present in the animal during vitamin B_{12} deficiency, as might be deduced from the defective ability of the deficient animal to utilize carbohydrate (Ling and Chow, '54), this would explain in part the lowered nucleic acid contents of the liver associated with the deficiency. The role of vitamin B_{12} in desoxyribose and purine synthesis by *Lactobacillus leichmannii* [11] may also have significance in animal metabolism. Further studies are now in progress to test this hypothesis.

SUMMARY

The growth rate, liver DNA and RNA levels, and liver regeneration ability after partial hepatectomy of rats fed an iodinated casein basal diet deficient in vitamin B_{12} were lower than those of supplemented controls.

In subsequent experiments, an uncomplicated vitamin B_{12} deficiency was produced in rats born from mothers fed the vitamin B_{12}-deficient diet without iodinated casein during gestation and lactation. The growth rate and liver nucleic acid composition of these rats fed the basal ration were significantly lower than those for the controls fed supplemented

[10] Wang, H., N. Maynard, W. T. Wong and B. S. Schweigert, unpublished data, Swift, H., E. Rasch, W. T. Wong and B. S. Schweigert, unpublished data.

[11] Downing, M., and B. S. Schweigert, unpublished data.

rations. A syndrome characterized by presence of porphyrin whiskers and scaly feet was also noted in the deficient rats.

The addition of dried liver to the non-iodinated casein ration did not improve the growth-promoting activity of vitamin B_{12} when the latter was present at a level of 50 μg/kg ration. However, inclusion of 4 or 10% of dried liver in the diet of the female rats, and 10% of liver in the diet of male rats, significantly increased the hemoglobin content of the blood ($P < 0.05$) as compared with that observed when the vitamin B_{12}-deficient diet was fed. The significance and implications of these findings were discussed.

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THE DIETARY FAT LEVEL IN THE NUTRITION OF THE RABBIT

EDWARD J. THACKER

U. S. Plant, Soil and Nutrition Laboratory, Agricultural Research Service, Ithaca, New York

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Experimental work in this laboratory has involved the use of whole cow's milk as the primary source of nutrients for the rabbit. This diet, although supplemented with all the known accessory food factors, resulted in a low rate of growth and in fatty degeneration or cirrhosis of the liver (Thacker and Ellis, '48). To facilitate the study of the apparent nutritional inadequacy of a milk diet for the rabbit, a synthetic diet was formulated that approximated the level of protein, fat and carbohydrate in whole milk. Unsatisfactory results with this diet raised the question of the role of fat in the nutrition of the rabbit and the possible detrimental effect of high levels of dietary fat on the health of the animal. The purpose of this investigation was to study the effect of fat levels in synthetic diets on the nutrition of the rabbit.

EXPERIMENTAL PLAN

The effect of the level of fat in purified diets was studied with Dutch belted rabbits 4 to 5 weeks of age in a randomized block design with 6 treatments and 9 replications under ad libitum feeding conditions.

The treatments consisted of 5 purified diets containing 5, 10, 15, 20 or 25% of fat and a stock diet.[1] The composition of the purified diets, and the nitrogen, ether extract, and gross energy (bomb calorimetry) content of all diets are shown in

[1] G. L. F. rabbit pellet.

table 1. The vitamin supplement was in proportion to the caloric density of the diets. Thus, the 5, 10, 15 and 20% fat diets were supplemented with 75, 81, 87 and 93%, respectively,

TABLE 1

Composition of diets [1]

CONSTITUENT	EXPERIMENTAL DIET DESIGNATED BY PERCENTAGE OF FAT				
	5	10	15	20	25
	%	%	%	%	%
Ruffex [3]	10	10	10	10	10
Casein, crude [4]	25	25	25	25	25
Dextrin [5]	54.9	49.9	44.9	39.9	34.9
Hydrogenated vegetable oil [6]	3	8	13	18	23
Corn oil [7]	2	2	2	2	2
Macro minerals [8]	5	5	5	5	5
Minor minerals [9]	0.1	0.1	0.1	0.1	0.1

THE VITAMIN MIXTURE SUPPLIED THE FOLLOWING PER 100 GM OF THE 25% FAT DIET [2]	
Water-soluble vitamins	
Thiamine	0.07 mg
Riboflavin	0.6 mg
Ca Pantothenate	1.5 mg
Pyridoxine	0.7 mg
Niacin	20.0 mg
Choline	100.0 mg
Betaine	100.0 mg
Inositol	10.0 mg
p-amino-benzoic acid	0.2 mg
Folic acid	0.10 mg
Biotin	0.05 mg
B_{12}	5 μg
Fat-soluble vitamins	
Vitamin A palmitate	666 I.U.
Calciferol	0.02 mg
Alpha tocopherol	7.5 mg
Menadione	0.075 mg

ANALYSES OF DRY MATTER						*Stock* [10]
Nitrogen, %	3.72	3.64	3.66	3.67	3.85	3.35
Ether extract, %	5.31	10.76	16.17	21.30	26.31	3.77
Cal/gm	4.26	4.73	4.97	5.09	5.40	4.18

[1] Designation of the manufacturer of the food materials used in this investigation does not indicate endorsement of the product by the U.S.D.A.

[2] The 5, 10, 15 and 20% fat diets were supplemented with 75, 81, 87, 93%, respectively, of the vitamins added to the 25% fat diet.

[3] Fisher Scientific Company.

[4] National Casein Sales Company, Chicago, Illinois.

[5] White Dextrin, N.F.V., Merck and Co., Inc.

[6] Primex, Proctor and Gamble, Cincinnati, Ohio.

[7] Mazola, Corn Products Refining Company.

[8] Hawk-Oser salt mixture.

[9] $FeC_6H_5O_4 \cdot 1\frac{1}{2}\ H_2O$ — 453.85, $CuSO_4 \cdot 5H_2O$ — 28.15, $MnSO_4 \cdot H_2O$ — 16.50, KI — 1.50 gm per kilogram.

[10] G. L. F. Rabbit Pellets, G. L. F., Ithaca, New York.

of the vitamins added to the 25% fat diet. Since the purified diets were fed in powdered form, the stock diet of rabbit pellets was pulverized in a hammer mill to give a feed of similar texture.

The animals were individually caged, maintained on raised wire screen floors, and were fed and watered daily. The food consumption was recorded daily and the rabbits were weighed weekly. Blood hemoglobin was determined during the 5th, 10th and 15th weeks of the experimental period.

The digestibility of dry matter, nitrogen, ether extract and the nitrogen balance were determined with two replicates of rabbits at 8, 10, 12, 14 and 15 weeks of age. A preliminary period of 7 days on a constant food intake preceded a collection period of 7 days. The urine and feces were collected daily. The urine and feces were analyzed for nitrogen, the feces for moisture and ether soluble substances by standard A. O. A. C. methods ('50). The rabbits were sacrificed at the conclusion of the 15-week experimental period, autopsied, and the livers were excised and weighed.

RESULTS

Five animals did not survive the experimental period (table 2). Two animals were lost from the 5% fat treatment: one died at 27 days in an emaciated condition with intestinal hyperemia; the other died at 54 days in an emaciated condition. The non-surviving animal from the 20% fat diet suffered an accidental injury. The rabbit from the 25% fat diet died at 97 days with a ruptured stomach, the cause for which was not apparent. The animal which died after 69 days on the stock diet was emaciated.

The rabbits receiving the 10, 15, 20 and 25% fat diets had similar gains in body weight and the gain of the rabbits on the stock diets was not significantly different from the gain of the rabbits receiving the above purified diets (table 2). The animals receiving the diet containing 5% of fat gained approximately 200 gm less ($P \leq 0.05$) than those receiving the diets with higher fat levels.

If the datum from a rabbit with an erratic growth curve is excluded from the statistical analysis, a highly significant difference among treatments results. The increased body gain observed when the rabbits were fed the 10, 15, 20 or 25% fat diet is highly significant ($P \leq 0.01$) when compared to the

TABLE 2

Average body weight, feed, and energy consumption and liver weight of rabbits fed ad libitum

DIET	NO. OF ANIMALS		BODY WEIGHT		GAIN PER GRAM FEED	KCAL. PER GRAM GAIN	LIVER WEIGHT AS PERCENTAGE OF BODY WEIGHT
	Initial	Final	Initial	Gain			
			gm	*gm*	*gm*		
5% Fat	9	7	353	1034	0.30	14.3	3.24
10% Fat	9	9	360	1246	0.33	14.4	3.85
15% Fat	9	9	355	1286	0.35	14.2	2.80
20% Fat	9	8	374	1186	0.35	14.7	2.75
25% Fat	9	8	357	1243	0.38	14.2	2.88
Stock	9	8	362	1119	0.21	19.6	2.37

TABLE 3

Apparent digestibility of dry matter, ether extract, protein of rabbits fed ad libitum

DIET	DRY MATTER	ETHER EXTRACT	CRUDE PROTEIN
	%	%	%
5% Fat	88 ± 1.2 [1]	97 ± 0.3	93 ± 1.9
10% Fat	91 ± 0.1	99 ± 0.1	95 ± 1.3
15% Fat	91 ± 0.8	99 ± 0.1	95 ± 0.3
20% Fat	90 ± 0.6	99 ± 0.4	93 ± 1.1
25% Fat	90 ± 0.5	99 ± 0.1	96 ± 1.2
Stock	74 ± 1.3	91 ± 0.8	79 ± 1.8

[1] Standard error.

gain made by the animals receiving the 5% fat or the stock diet. The rabbits fed the stock diet gained more than those fed the 5% fat diet (significant when $P \leq 0.05$).

The efficiency of utilization of feed for body gain increased in general as the fat content of the purified diets increased

(table 2). Nearly twice as much of the stock diet was required to produce a gram of gain as was required of the 25% fat diet.

The livers of the rabbits receiving the purified diets were light in color, as contrasted to those from the rabbits fed the stock diet.

The average apparent digestibility (table 3) of the dry matter, ether extract, and crude protein of the purified diets was not affected by age of dietary fat level.

DISCUSSION

When rabbits were fed to appetite, a 10 to 25% fat concentration in the diet supported a 23% greater body gain than did the diet with 5% fat. However, the greater gain was accomplished at the expense of a greater caloric consumption as evidenced by the similar number of calories required per gram of gain.

Ad libitum feeding tests with rats have given comparable results (Hoagland and Snider, '40; Hoagland, '41). Deuel and co-workers ('47) found that caloric consumption tended to be greater with increasing dietary fat level. In the study with rabbits reported here, the increased caloric consumption associated with the higher fat levels was not only a result of the higher caloric density of the diets but also of an increase in the quantity of food consumed. This was first evident with the use of the 10% fat diet. When the dietary level of fat was doubled, feed consumption increased 10% and caloric consumption 22%. Above a level of 10% fat, feed consumption gradually decreased as the level of dietary fat increased with a concomitant rise of 2 to 3% in calories consumed.

The results with the rat and rabbit suggest that fat influences the acceptability of the diet. Swift ('52) reported that rats fed a 2% fat diet refused food more often than did rats fed higher levels of fat. Maynard and Rasmussen ('42), using a paired feeding technique observed that rats receiving a stock diet with a normal level of fat practically always limited the food intake of rats receiving the same diet with added fat. Scott and Verney ('48) using a self-selection technique re-

ported that rats consumed more calories as fat than they did as protein or sucrose.

The effect of dietary fat on body gain, lactation performance (Maynard and Rasmussen, '42; Maynard, Loosli and McCay, '41; Bender and Maynard, '32) and energy utilization (Swift, '52) is demonstrable with isodynamic feeding where the low-fat ration contains 1 to 2% of fat. In rat (Hoagland et al., '52), steer (Willey et al., '52), sheep (Swift et al., '48), and rabbit studies such effects of dietary fat were not evidenced when the low-fat ration contained 3 to 5% ether extract. On the other hand, with ad libitum feeding, the concomitant effect of dietary fat levels would appear to be that of increasing caloric consumption with increasing fat levels and an associated increase in body gain. This effect with the rabbit is apparent when the low fat diet contains as much as 5% of fat.

SUMMARY

Under conditions of ad libitum feeding, rabbits fed a purified diet that contained at least 10% of fat made greater body gains than did animals fed a similar diet that contained 5% of fat or when fed a commercial rabbit ration. It is probable that the increased acceptability of the diets with higher levels of fat was a significant factor in the increased gains observed with ad libitum feeding.

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THE RELATIONSHIP OF VITAMIN B_6 TO PROTEIN METABOLISM DURING PREGNANCY IN THE RAT [1]

MARGARET L. ROSS [2,3] AND RUTH L. PIKE
Department of Foods and Nutrition, The Pennsylvania State University, University Park

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In a study on the effects of a vitamin B_6 deficiency during reproduction, Nelson and Evans ('48, '51) reported that poor reproductive performance occurred only when there was depletion of maternal vitamin B_6 stores prior to mating. This evaluation of reproductive performance was based upon the number and size of the young produced and upon the incidence of resorption. We were interested in studying the effects of a vitamin B_6 deficiency during gestation upon the maternal organism as well as the offspring, and to determine whether indications of adverse effects might be observable without a prior depletion period. Further, we were interested in observing the relation of this vitamin B_6 deficiency to maternal protein metabolism during reproduction. The factors to be reported in this paper are maternal weight gain, nitrogen retention and hepatic nitrogen and moisture, in addition to the number of young per litter, the weights of the individual young and the number of resorptions. A subsequent report will present data on the maternal serum proteins and nonprotein nitrogen at term.

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[2] Taken in part from a dissertation submitted to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirement for the Doctor of Philosophy degree.

[3] Mary Swartz Rose fellow, 1953–54; present address: Division of Home Economics, Iowa State College, Ames, Iowa.

EXPERIMENTAL METHOD

Female albino rats of the Sprague-Dawley strain were maintained on laboratory chow [4] until they attained a weight of approximately 200 gm. Estrous cycles were followed by means of daily vaginal smears. When regular cycles were established the animals were divided into two main groups, one of which was maintained on laboratory chow until the day mating was confirmed, and the other subjected to a prior depletion period (vitamin B_6-deficient basal ration plus 0.5 mg % of desoxypyridoxine) for at least 7 days before mating. The length of the depletion period varied from 7 to 30 days because of irregularity of estrous cycles in animals subjected to vitamin B_6 deficiency. The morning mating was confirmed by the presence of sperm in the vaginal smear was considered the first day of pregnancy. The animals were then placed in individual cages and those from both the non-depleted and depleted groups received the basal ration plus one of the 5 dietary supplements shown in table 1. These diets were fed ad libitum and weekly food consumption records kept. Maternal weight gains were recorded daily. Vaginal smears were made daily also to observe any indications of interruption of pregnancy or resorption.

On the 22nd day the animals were sacrificed and the livers removed, weighed and frozen for later analyses. The young were removed and the number of live young per litter, the weights of the individual young and the number of resorptions were recorded.

The nitrogen content of weighed samples of maternal liver was determined by a macro-Kjeldahl method. Moisture content was determined by drying slices of frozen liver to constant weight under an infra-red lamp.

The data were analyzed statistically by means of analysis of variance. Comparisons were made between the two main groups to test for the effects of vitamin B_6 deficiency prior to pregnancy, and within each main group to test for the

[4] Purina.

TABLE 1

Composition of experimental diets

BASAL RATION				SUPPLEMENTS [1]		
	%	*Vitamin mixture* [2]	*mg*	Diet	Desoxy-pyridoxine	Pyridoxine
Casein, vitamin test [3]	26	Thiamine	2.0		*mg %*	*mg %*
Sucrose and vitamin mixture	9.85	Riboflavin	2.0	1	0.5	0
Cornstarch	34	p-Aminobenzoic acid	200.0	2	0	0
Hydrogenated fat [4]	19	Niacin	10.0	3	0	0.4
Corn oil [5]	5	Pantothenic acid	8.0	4	0	0.8
Salt mixture [6]	4	Biotin	0.04	5	0	1.2
Agar	2	Inositol	400.0			
L-cystine	0.15	B_{12} triturate [7]	4.0			
Vitamin ADE mixture [8]	+	Folic acid	0.4			
		Choline	400.0			
		Naphthoquinone	1.0			

[1] Incorporated into diet.
[2] Made up to 9.85 gm with sucrose.
[3] Labco.
[4] Crisco.
[5] Mazola.
[6] Hawk-Oser — Science, *74:* 369, 1931.
[7] One-tenth per cent trituration of crystalline B_{12} in mannitol (Merck).
[8] ADE mixed in corn oil contained 5,000 I.U. of A, 400 I.U. of D_3 and 10 mg of alpha tocopherol in 2 drops and was administered 2 drops per rat every three days.

effect of the level of vitamin B_6 during pregnancy. All analyses were corrected for disproportionality among the groups.

RESULTS AND DISCUSSION

Maternal weight gain. An increase in maternal weight gain is generally observed during the gestation period since, in addition to the growth and development of the fetus, there is enlargement of the uterus and mammary glands and the development of placental tissues. Maternal weight gain as a criterion for the evaluation of a diet during pregnancy has limitations, however, because neither the magnitude nor the composition of the most desirable gain are known.

TABLE 2

Average maternal weight gain

PYRIDOXINE SUPPLEMENT	NON-DEPLETED		DEPLETED	
	No. of animals	Total gain	No. of animals	Total gain
mg %		*gm*		*gm*
0 [1]	10	61 ± 7 [2]	8	38 ± 3
0	10	67 ± 4	8	55 ± 5
0.4	10	111 ± 6	8	85 ± 6
0.8	10	115 ± 6	7	90 ± 8
1.2	11	109 ± 4	8	99 ± 5

[1] Plus 0.5 mg % of desoxypyridoxine.

[2] Standard error of the mean.

In reproduction studies with rats a weight gain of 100 gm or more has been considered one indication of good reproductive performance. Nelson and Evans ('51) have shown that vitamin B_6 is required for satisfactory reproduction and that its lack causes lowered maternal weight gain.

In the present study the maternal weight gains during the gestation period differed with the level of dietary pyridoxine, the presence of desoxypyridoxine in the diet, and the state of the maternal pyridoxine stores prior to mating (table 2).

An analysis of variance showed that the differences due to both diet and depletion were significant ($P = 0.01$). There

was also an interaction between the effects of the diets and prior depletion ($P = 0.01$); that is, the effects produced by desoxypyridoxine or the level of pyridoxine in the ration were different in the non-depleted and depleted animals. Although there were significant differences in weight gains between the non-depleted and depleted groups receiving pyridoxine, the differences observed on the three levels within each group were not significant.

Average maternal weight gains of over 100 gm were observed in the animals not depleted of their vitamin B_6 stores prior to mating and maintained during the gestation period on diets containing pyridoxine. Since the differences in the weight gains observed for these animals were not significant, it appears that the 0.4 mg % level is an adequate pyridoxine intake for the rat during pregnancy if maternal weight gain is used as the only criterion. The maternal weight gains observed by Nelson and Evans ('53) with 0.5 mg % of pyridoxine support this interpretation.

Depletion of the maternal vitamin B_6 stores prior to mating exerted a marked effect on weight gain. The smallest weight gain occurred in the group receiving desoxypyridoxine and increased linearly as the level of pyridoxine was increased. This linear increase in average weight gain demonstrated by the depleted animals receiving increasing amounts of pyridoxine appears to indicate that the maternal organism may be able to overcome some of the effects of prior depletion if the level of pyridoxine during gestation is sufficiently high. Possibly an increase in pyridoxine above 1.2 mg % could have completely counteracted the effects of depleting the maternal vitamin B_6 stores before mating. This suggestion is supported by the data presented by Nelson and Evans ('51) who reported that a 5.0 mg % pyridoxine supplement to the diet of depleted animals on the first day of gestation counteracted all the deleterious effects of a prior depletion period.

The animals receiving the desoxypyridoxine supplement during gestation in both the depleted and non-depleted groups

in this study gained more weight than similar groups of animals reported by Nelson and Evans ('51). However, there were two fundamental differences in the basal rations which may account for this: the higher non-protein calorie level and the inclusion of corn oil in the basal ration used in the present investigation.

Since a relationship between calorie intake and protein utilization during pregnancy has been reported by Oldham and Sheft ('51) and by our laboratory (Pike, Suder and Ross, '54), and since evidence has been presented indicating that the inclusion of corn oil in the ration is necessary for normal reproduction (Kummerow, Pan and Hickman, '52), it would appear that the higher maternal weight gains observed on the desoxypyridoxine-supplemented rations in this study were due to the more adequate basal ration.

Failure to gain weight and disturbances in protein and fat metabolism have been reported for the vitamin B_6-deficient growing rat (Witten and Holden, '52; Beare, Beaton and McHenry, '53; Beaton et al., '53a, '53b, '54b; Olsen and Martindale, '54), but no studies have been reported on the effects of a lack of vitamin B_6 on protein and fat metabolism during gestation. A relationship between pyridoxine and several of the hormones in the growing rat has been reported by Beaton, Beare and McHenry ('52). Since pregnancy is normally a period of growth accompanied by hormonal changes, and since disturbances in hormonal secretions have been observed in vitamin B_6-deficient pregnant rats [decreased secretion of gonadotrophins by Nelson, Lyons and Evans ('51, '52); and cessation of estrous in the present study], it is suggested, therefore, that the low weight gains observed in the pyridoxine-deficient animals in this study may have been due to disturbances in both protein and fat metabolism and to an hormonal imbalance.

Food intake. In studies with pyridoxine-deficient diets decreases in food intake of approximately 50% have been reported (Nelson and Evans, '51; Beaton et al., '53a; Beaton, Smith and McHenry, '53). Pair-fed control animals have

been used in these and other studies (Sure and Easterling, '49; Olsen and Martindale, '54) to determine if the effects observed were due to inanition or to lack of pyridoxine. The results of these studies indicate that a lack of pyridoxine decreases the amount as well as the utilization of the food ingested.

The data presented in table 3 show that there was little difference in food consumption between the depleted and non-depleted groups. The food consumption in each group was highest for the animals on the pyridoxine-containing

TABLE 3

Food intake, nitrogen intake and percentage nitrogen retention
Average totals for second and third weeks of pregnancy

PYRIDOXINE SUPPLEMENT	NON DEPLETED [1]			DEPLETED [2]		
	Intake		Nitrogen retention	Intake		Nitrogen retention
	Food	Nitrogen		Food	Nitrogen	
mg %	*gm*	*mg*	*%*	*gm*	*mg*	*%*
0 [3]	126 ± 6 [4]	5058 ± 226	15.3	115 ± 6	4582 ± 246	4.1
0	132 ± 6	5210 ± 257	15.6	133 ± 5	5283 ± 181	8.1
0.4	171 ± 7	6693 ± 288	26.0	166 ± 7	6527 ± 262	16.2
0.8	167 ± 5	6641 ± 188	24.2	168 ± 6	6660 ± 227	11.6
1.2	162 ± 4	6369 ± 166	24.6	178 ± 7	7052 ± 270	14.4

[1] Ten to 11 animals per group.
[2] Seven to 8 animals per group.
[3] Plus 0.5 mg % of desoxypyridoxine.
[4] Standard error of the mean.

rations, lower on the pyridoxine-free diet and reduced further on the desoxypyridoxine supplement. Statistical analysis showed that prior depletion had no significant effect on the quantity of food consumed; however, the differences in food intakes on the desoxypyridoxine-supplemented and pyridoxine-free diets were significantly lower than those on the pyridoxine-containing diets ($P = 0.01$).

Nitrogen retention. Nitrogen retention is normally expected to occur during the gestation period. In addition to the nitrogen that is retained by the fetus, nitrogen is also required for the growth of the uterus, placental tissues and

mammary glands. Balance studies on pregnant women have shown that the maternal body retains a considerable excess of nitrogen beyond that required for the fetus and its adnexa (Hunscher et al., '33, '35). Reproduction studies with rats in our laboratory and those reported by Beaton et al. ('54a) have demonstrated that nitrogen retention increases throughout pregnancy with the greatest storage occurring during the third week of gestation, the period of rapid fetal growth.

The influence of the non-protein calorie intake on the utilization of protein has been reported by numerous investigators (Benditt et al., '48; Geiger, '51; Swanson, '51). The importance of this relationship during pregnancy has been demonstrated in human subjects by Oldham and Sheft ('51) and in rats by Pike, Suder and Ross ('54) who showed a limiting effect of low non-protein calories on nitrogen retention.

In the present study nitrogen retentions were compared for only the second and third weeks of pregnancy. The first week involved a change in diet for all of the animals except those in the depleted group receiving the desoxypyridoxine supplement and was, therefore, considered an adjustment period.

The average nitrogen retentions of the animals on all of the diets in the depleted group were lower than on the corresponding diets in the non-depleted group. Since the nitrogen intakes did not differ, the percentage of the dietary nitrogen that was retained was considerably lower in the depleted group as is shown in table 3.

In each group those animals receiving desoxypyridoxine had the lowest nitrogen retentions. The retentions were higher in both groups on the pyridoxine-free diet. When pyridoxine was added to the ration the percentage of nitrogen retained was markedly increased.

Statistical analysis of the data showed that depletion had a significant effect on nitrogen retention ($P = 0.01$). In both groups the differences in nitrogen retention on the desoxypyridoxine-supplemented and pyridoxine-free rations were

significantly lower than on the pyridoxine-supplemented rations ($P = 0.01$). However, the level of pyridoxine supplementation produced no significant effect on nitrogen retention within the two main groups.

The decreased nitrogen retentions in both the second and third weeks of pregnancy of all the animals receiving the desoxypyridoxine-supplemented and pyridoxine-free rations during gestation may have been due to the decreased food consumption and, thus, the lower calorie and nitrogen intake of these animals. However, the calorie and nitrogen intakes of the animals receiving pyridoxine were comparable, but the nitrogen retentions were lower in the depleted group. Therefore, it seems valid to assume that the decreased nitrogen retentions observed were the result of an impairment in nitrogen metabolism due to the lack of pyridoxine which, in the pyridoxine-deficient groups, was superimposed upon the limitations induced by a reduction of calorie intake. The decreased nitrogen retentions of vitamin B_6-deficient animals compared to those of pair-fed controls and the increased retentions of ad libitum controls which have been reported by Beaton et al. ('53a) support this assumption.

Since Carter and Phizackerley ('51) have demonstrated that no alteration occurs in the absorption of protein, fat and carbohydrate in the vitamin B_6-deficient rat, and since this observation has been confirmed for protein absorption by Beaton et al. ('53a), the decreased nitrogen retentions would appear to be due to a disturbance in amino acid metabolism. Vitamin B_6 has been shown to be necessary for transamination, deamination, desulfhydration and decarboxylation, as well as for the normal metabolism of tryptophan, all fundamental enzymatic reactions and all shown to be disturbed in vitamin B_6 deficiency. Therefore, if there is an interference in amino acid metabolism, protein synthesis and the accompanying nitrogen retention which is normal during pregnancy may be disturbed. In addition, interrelated reactions in intermediary metabolism involving carbohydrate and fat may also be affected.

Liver weight, moisture and nitrogen content. The capacity of the liver to change in size and protein content under various stress conditions including the stress of pregnancy has been established. The livers of rats subjected to a vitamin B_6 deficiency have been shown to increase in size thus producing an increase in the proportion of liver weight to body weight (Beaton et al., '53b; Olsen and Martindale, '54).

There was no marked difference observed in this study in the average moisture content of the livers (table 4). Analysis of variance showed no significance for the effects of diet or depletion alone, but an interaction effect of slight significance was shown ($P = 0.05$); that is, prior depletion had some influence on the response to the diet.

Since the weights of the animals at term were different in the various groups, liver weights and nitrogen contents were calculated on the basis of 100 gm of body weight for comparative purposes. On this basis, increased ratios of liver weight to body weight were observed for all of the animals subjected to prior depletion and for the non-depleted animals maintained on the desoxypyridoxine supplement during pregnancy. Statistical analysis of the data showed that the increased ratios were significant due to the effects of depletion ($P = 0.01$) and to the effects of the desoxypyridoxine supplement ($P = 0.05$).

When nitrogen content was calculated on the basis of milligrams of nitrogen per weight of dry liver per 100 gm of body weight, the livers of the animals maintained on the desoxypyridoxine-supplemented ration, in both the non-depleted and depleted groups, had the highest nitrogen content. Statistically, this was of slight significance ($P = 0.05$). However, a statistically significant difference was observed due to the effect of depletion ($P = 0.01$). No significance was found for the differences observed in the liver nitrogen per 100 gm of body weight on the three levels of pyridoxine supplementation within each main group.

The significantly higher ratios observed in the weight and nitrogen content of the liver per 100 gm of body weight for

TABLE 4

Average moisture, weight and nitrogen content of maternal livers

PYRIDOXINE SUPPLEMENT	NON-DEPLETED [1]			DEPLETED [2]		
	Moisture	Weight [3] Per 100 gm body weight	Nitrogen [4] Per 100 gm body weight	Moisture	Weight [3] Per 100 gm body weight	Nitrogen [4] Per 100 gm body weight
mg %	%	*gm*	*mg*	%	*gm*	*mg*
0 [5]	72.6 ± 0.3 [6]	3.539 ± 0.181	388 ± 14	73.4 ± 0.4	3.784 ± 0.102	446 ± 9
0	73.9 ± 0.4	3.002 ± 0.139	366 ± 17	72.7 ± 0.3	3.392 ± 0.099	395 ± 8
0.4	72.9 ± 0.4	3.083 ± 0.079	365 ± 10	73.5 ± 0.3	3.338 ± 0.114	401 ± 10
0.8	73.4 ± 0.1	3.084 ± 0.107	367 ± 10	73.7 ± 0.4	3.529 ± 0.096	417 ± 11
1.2	73.7 ± 0.3	3.192 ± 0.015	383 ± 13	73.6 ± 0.3	3.392 ± 0.068	415 ± 8

[1] Ten to 11 animals per group.
[2] Seven to 8 animals per group.
[3] Wet weight basis.
[4] Dry weight basis.
[5] Plus 0.5 mg % of desoxypyridoxine.
[6] Standard error of the mean.

TABLE 5

Average litter size, fetal weight, resorptions and length of depletion

PYRIDOXINE SUPPLEMENT	NON-DEPLETED [1]			DEPLETED [2]			
	No. of live young	Fetal weight	No. of resorptions	No. of live young	Fetal weight	No. of resorptions	No. of depletion days
mg %		*gm*			*gm*		
0 [3]	9.8 ± 0.7 [4]	4.3 ± 0.5	0.8 ± 0.3	10.1 ± 0.7	2.8 ± 0.1	1.0 ± 0.3	12 ± 1
0	8.4 ± 0.9	4.7 ± 0.1	1.7 ± 0.7	9.6 ± 1.0	3.3 ± 0.2	2.0 ± 0.8	14 ± 2
0.4	10.5 ± 0.9	5.2 ± 0.1	0.1 ± 0.1	8.4 ± 1.0	4.3 ± 0.2	0.3 ± 0.2	14 ± 2
0.8	9.3 ± 1.0	5.5 ± 0.2	0.1 ± 0.1	8.4 ± 1.5	3.5 ± 0.5	2.3 ± 0.7	19 ± 3
1.2	8.3 ± 0.3	5.3 ± 0.2	0.4 ± 0.1	8.9 ± 0.8	4.0 ± 0.2	1.8 ± 0.7	16 ± 3

[1] Ten to 11 animals per group.
[2] Seven to 8 animals per group.
[3] Plus 0.5 mg % of desoxypyridoxine.
[4] Standard error of the mean.

the animals receiving desoxypyridoxine, either prior to mating or during the gestation period, confirm the work of other investigators (Beaton et al., '53b; Olsen and Martindale, '54), but the results of this study do not support the suggestion of the former investigators that the synthesis of liver protein is independent of vitamin B_6 intake. It would appear, rather, that vitamin B_6 exerts some control over the extent to which liver protein synthesis proceeds. An inspection of the present data shows that incorporation of desoxypyridoxine into the maternal diet resulted in a higher ratio of liver weight and nitrogen content per 100 gm of body weight, whereas pyridoxine supplementation of the depleted animals during the gestation period caused a lowering of the ratio of liver weight and nitrogen content per 100 gm of body weight. The decreased ratios, however, did not attain the lower values observed for the non-depleted animals maintained on the corresponding diets. This is a further indication that the maternal organism cannot completely overcome the effects of depletion of the vitamin B_6 stores prior to mating.

The data indicate that there is a definite influence of vitamin B_6 on liver protein synthesis during pregnancy. It is postulated that this effect may be due to the influence of vitamin B_6-dependent enzyme systems or perhaps to hormonal changes. The alterations in the activity of some of the liver enzymes involved in protein metabolism observed in vitamin B_6-deficient rats (Beaton, '54; Beaton et al., '54b), the hormonal changes reported in vitamin B_6-deficient pregnant animals (Nelson, Lyons and Evans, '51, '52), the cessation of estrous observed in the present study, and the hypertrophy of the adrenal glands of vitamin B_6-deficient animals (Butler and Morgan, '54; Olsen and Martindale, '54) give some support to this hypothesis.

The similarity of the response of the animals in the non-depleted group receiving the pyridoxine-free diet to that of the animals receiving the pyridoxine supplements may be an indication that either the pyridoxine deficiency was not severe enough to affect liver protein synthesis, or that avail-

able extra-hepatic stores of vitamin B_6 were mobilized to the liver under the stress of pregnancy.

Litter size, fetal weight and resorptions. In addition to maternal weight gain and nitrogen retention, the general criteria used in evaluating the adequacy of the diet for successful reproduction in the rat are the number of live young per litter and the fetal weight. Nelson and Evans ('48, '51) have considered, as a further criterion, the incidence of resorption.

The results obtained in this study indicate that the number of young per litter is not a valid criterion. Statistical analysis of the data showed that there were no significant differences in the number of young produced by the mothers in the non-depleted and the depleted groups nor among the animals maintained during pregnancy on the different diets (table 5). Although these findings confirm previous work in this laboratory, they are in contrast to the findings of Nelson and Evans ('48). These investigators observed a tendency to decreased number of young when animals were maintained on pyridoxine-deficient diets and to increased number of young when 0.5 mg % of pyridoxine was incorporated into the diet of depleted animals on the first day of the gestation period.

Average fetal weights of over 5 gm are generally accepted as one evidence of the adequacy of the diet for reproduction (Sica and Cerecedo, '48). In all of the diet groups in this study the average weights of fetuses from non-depleted mothers were higher than those from mothers that were depleted prior to mating. The young from all of the animals maintained on the desoxypyridoxine-supplemented and the pyridoxine-free diets during pregnancy, and even those from the depleted animals that received pyridoxine supplements during pregnancy, were below the generally accepted viable weight. Therefore, judged on the basis of average fetal weight, only the non-depleted animals receiving pyridoxine demonstrated good reproductive performance. Statistical analysis of the data showed significance for the effects of depletion

and for the effects of the desoxypyridoxine-supplemented and the pyridoxine-free diets ($P = 0.01$). No significant differences were shown due to the three levels of pyridoxine within each of the two main groups.

The significantly lower average weights of the young from the depleted animals and from those in the non-depleted group receiving the desoxypyridoxine-supplemented and pyridoxine-free diets paralleled the lowered nitrogen retentions of the mothers. This would appear to indicate that the disturbances in nitrogen metabolism brought about by a lack of pyridoxine were reflected in the weight of the offspring produced. The findings of Suder ('52) that the weight of the fetus is correlated directly with the nitrogen content support this interpretation.

A significant finding in this study was that the average weight of the young from the depleted mothers maintained during gestation on diets containing pyridoxine were as low as or lower than those of the young from the non-depleted animals maintained on the desoxypyridoxine-supplemented diet during gestation. This is in direct contrast to the report by Nelson and Evans ('48) that the weight of the young was not affected adversely if pyridoxine was incorporated into the diet of depleted animals on the first day of gestation.

When average fetal weight is used as the criterion, the reproductive performance of the non-depleted animals maintained on the pyridoxine-supplemented diets appeared to be successful. The tendency to slightly heavier young found on the 0.8 mg % pyridoxine supplement suggests that this may be a satisfactory level for reproduction in the rat if average fetal weight is used as the criterion.

If the additional criterion of incidence of resorption is used to evaluate the diets employed in this study, only the non-depleted animals receiving 0.4 mg % and 0.8 mg % of pyridoxine exhibited satisfactory reproductive performance. In all cases, the incidence of resorption was greater in the depleted group than on the corresponding diets in the non-depleted group. The depleted animals on the high pyridoxine

supplements had an average number of resorptions as great as those in animals on the pyridoxine-deficient diets. The incidence of resorption was found to have more statistical significance due to the effects of depletion ($P = 0.01$) than to diet ($P = 0.05$). This is in further contrast to the findings of Nelson and Evans ('48). Although they observed a high incidence of resorption in depleted animals maintained on a desoxypyridoxine-supplemented diet during gestation, they reported no resorptions occurring when pyridoxine was given to depleted animals during gestation.

The data obtained on the offspring in this study suggest that pyridoxine in the diet before mating is as essential as pyridoxine in the diet during gestation. Further, it supports the hypothesis that the condition of the maternal organism prior to the inception of pregnancy plays a critical role in the course of pregnancy and its outcome.

SUMMARY AND CONCLUSIONS

The effects on both the maternal organism and the offspring of depletion of the maternal vitamin B_6 stores prior to mating and of varying levels of pyridoxine in the diet during gestation were investigated.

Average maternal weight gains of over 100 gm were observed in animals not depleted of their vitamin B_6 stores prior to mating and maintained during the gestation period on diets containing pyridoxine. Depletion of maternal vitamin B_6 stores prior to mating exerted a marked effect on weight gain. The smallest weight gains occurred in the group receiving desoxypyridoxine and increased linearly as the level of pyridoxine was increased.

Prior depletion had no effect on food intake. Food consumption was highest for the animals receiving pyridoxine, lower on the pyridoxine-free diet and reduced further on the desoxypyridoxine supplement.

The percentages of dietary nitrogen retained were lower for all animals in the depleted group than for those on corresponding diets in the non-depleted group. Nitrogen reten-

tion was significantly lower on the desoxypyridoxine-supplemented diet in each group but the level of pyridoxine in the ration had no significant effect on nitrogen retention within the two main groups.

The data on maternal livers showed that there is a definite influence of vitamin B_6 on liver protein synthesis during pregnancy. It is postulated that this effect may be due to the influence of vitamin B_6-dependent enzyme systems or to hormonal changes.

Average fetal weights differed with the state of maternal vitamin B_6 stores prior to mating and with the level of pyridoxine in the ration during gestation. The young from non-depleted mothers were heavier than those from depleted mothers on corresponding diets. It is significant that the average weight of the young from depleted mothers maintained during gestation on pyridoxine-supplemented diets were as low as or lower than those from non-depleted animals maintained on the desoxypyridoxine-supplemented diet during gestation.

The data indicate that pyridoxine in the diet before mating is as important as pyridoxine in the diet during gestation; giving further support to the hypothesis that the condition of the maternal organism prior to the inception of pregnancy plays a critical role in the course of pregnancy and its outcome.

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PROTEIN AND AMINO ACID REQUIREMENTS OF THE GUINEA PIG

II. EFFECT OF AGE, POTASSIUM AND MAGNESIUM AND TYPE OF PROTEIN

HERBERT R. HEINICKE, ALFRED E. HARPER AND C. A. ELVEHJEM
Department of Biochemistry, University of Wisconsin, Madison

ONE FIGURE

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Previous work from this laboratory (Heinicke et al., '55) showed that the need for high levels of casein in purified diets for young guinea pigs is due to the high requirements of this species for a few specific amino acids, particularly arginine. The work reported in this paper extends these observations and shows that satisfactory responses to amino acid supplements can only be obtained when high levels of potassium and magnesium are provided. Responses to graded levels of amino acids and to different proteins have also been studied with older animals.

EXPERIMENTAL

The experimental procedures were similar to those previously described (Heinicke et al., '55). Young male guinea pigs were used in the first experiment and were continued in the second experiment to study the responses of older guinea pigs to graded levels of amino acids. Older animals from previous studies were used in the third experiment to determine the relationship of potassium and magnesium to

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growth responses obtained with amino acid supplements. These animals were then used in the last experiment to determine the relative value of different proteins in purified diets for guinea pigs. In switching from one experiment to another, a 3 to 5-day adaptation period was allowed, after which the animals were redistributed into groups of 5 with equal average weights.

The experimental plan for each experiment is indicated in the tables of results and in figure 1. The basal diet for all experiments, except the third one, was a 24% casein-sucrose ration similar to that previously described (Heinicke et al., '55) with the exception that the corn oil content was raised to 7.4% to reduce wastage and provide a ration with a better texture. The casein content was set at 24% to provide a level of protein [2] comparable to that found in guinea pig pellets.[3] The diets for experiment 3 contained graded levels of casein and were identical with those described in the previous paper. The amino acid supplements for both the sucrose and the dextrin groups were: with 20% casein, 1% glycine, 0.5% L-arginine-HCl, 0.3% DL-methionine, and 0.1% DL-tryptophan; with 25% casein, 0.5% L-arginine-HCl and 0.3% DL-methionine; with 30% casein, no supplements. The omission of 2.5% potassium acetate or 0.5% magnesium oxide or both was compensated for by increasing the carbohydrate.

RESULTS

Experiment 1, amino acid supplementation. The results of this experiment are presented in table 1. It can be seen that adding 0.5% of arginine to the basal diet produced a nearly three-fold increase in growth rate during the 5 weeks of period A. The addition of methionine alone to the basal diet was without effect, but the addition of methionine with arginine caused a greater growth response than did arginine alone. The 35% casein-sucrose diet supported satisfactory

[2] Kjehldahl analysis revealed that on a 16% basis, there was 83% protein in our alcohol-extracted casein.

[3] Rockland Guinea Pig Pellets, calculated on the basis of active components.

TABLE 1

Experiment 1

The effect of amino acid supplementation upon the growth of young, male guinea pigs fed 24% casein-sucrose diets [1]

	GROUP NO. AND DIET	DIETARY CHANGES [2] AND LENGTH OF PERIOD				
		Period A Control (5 weeks)	Period B, + 0.1% DL-tryptophan (2 weeks)	Period C,[3] + 0.5% A, + 0.3% M, + 0.1% T (1 week)	(12 days)	Period D,[3] + 0.1% L-arginine-HCl (10 days)
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*
1	Basal (24% casein-sucrose)	1.8 [4]	3.2	10.4	9.3	1.7
2	Basal + 0.5% L-arginine-HCl	5.2	5.1	7.9	8.4	3.5
3	Basal + 0.3% DL-methionine	2.0	1.8	8.4	8.1	2.5
4	Basal + 0.5% L-arginine-HCl + 0.3% DL-methionine	6.4	6.2	5.6	5.0	3.6
5	35% casein-sucrose	7.5	5.4	6.5		3.0
		[7.0, 3 wks. (5) / 8.0, 4 wks. (3)] [5]	(18 days)	(8 days)		

[1] Results listed as average gain in gm/day; av. starting wt. = 206 gm, 5 guinea pigs per group.

[2] A = L-arginine-HCl; M = DL-methionine; T = DL-tryptophan.

[3] All groups received identical 24% casein diets supplemented as shown in column headings.

[4] During the 5th week the gain was 3.0 gm/day.

[5] Two of the animals became sick and stopped growing after three weeks.

growth during this period. One animal died from this group and to equalize the groups, the poorest animal from each of the other groups was also removed after period A.

The results for period B show that the addition of tryptophan did not improve any of the 24% casein-sucrose diets and even slightly depressed the growth rate of the group receiving the 35% casein-sucrose diet. The results for period C again show the need for arginine and methionine by the remarkable responses that were obtained in groups 1, 2 and 3 when these amino acids were first added to diets that had been deficient in either one or both of them. Group 4, which now served as the control, because it had received these supplements throughout the previous periods, actually grew slightly less during this period, possibly because the animals were approaching the end of the phase of rapid growth. The improved growth rate of the animals of group 5 shows that 0.1% of tryptophan was no longer depressing growth under these conditions. The results for the last period show that 0.1% of arginine was insufficient to produce satisfactory growth responses with guinea pigs approximately three months old.

Experiment 2, graded levels of arginine and methionine. The results for this experiment are given in table 2. In period E, the group receiving 0.1% of arginine grew considerably better during the second week (4.5 vs. 3.3 gm/day). However, the growth rate was still better when the diet contained 0.3% of arginine.

In contrast to the positive responses obtained a few weeks earlier when 0.5% of arginine was given, the group receiving 0.6% of arginine showed an initial depression in growth rate. This was overcome and compensated for by the end of the experiment, and was similar to the situation observed when 0.5% of arginine was added to the diet of a group receiving 30% of casein with sucrose (Heinicke et al., '55). The possibility remains that in the presence of supplementary methionine (not included with the graded levels of arginine), excess arginine could have been metabolized more readily,

possibly as creatine or creatinine, thereby reducing or eliminating the initial growth depression.

The results for period F show that except for the first week, these animals (about 5 months old at the end of the experiment) did not respond to supplementary methionine in the presence of arginine. In fact, 0.3% of methionine, the level which previously caused a positive growth response, now seemed to depress growth. Since the presence

TABLE 2

Experiment 2

The effect of graded levels of L-*arginine-HCl and of* DL-*methionine with 0.3% of* L-*arginine-HCl upon the growth of older guinea pigs fed 24% casein-sucrose diets* [1]

Graded levels of arginine (no methionine)

ARGININE LEVEL		(Av. starting wt. = 571 gm)		
		0.1%	0.3%	0.6%
		gm/day	*gm/day*	*gm/day*
Period E	1 week	3.3	5.3	4.3
	17 days	3.9 (14 days)	5.2	5.4

Graded levels of methionine with 0.3% L-arginine-HCl

METHIONINE LEVEL		(Av. starting wt. = 637 gm)		
		0.0%	0.15%	0.30%
		gm/day	*gm/day*	*gm/day*
Period F	1 week	3.6	4.9	2.7
	25 days	4.6	4.9	2.4

[1] Responses listed as average gain in gm/day, 5 guinea pigs per group.

of one amino acid can modify the effect of another, more work will be needed to determine whether the growth depression of older guinea pigs was due, primarily to methionine, or to the combination of methionine and arginine.

Experiment 3, high levels of potassium and magnesium. The results for the group receiving 25% of casein with dextrin are presented graphically in figure 1. Since the results for the other 5 groups were qualitatively the same, they are not presented. The few differences that were obtained with the other groups will be discussed directly.

It can be seen that the animals immediately lost weight when the extra potassium and magnesium salts were omitted from the diet. In general, this effect was more pronounced in the sucrose groups and was from two to three times as great in groups receiving 30% of casein which lost an aver-

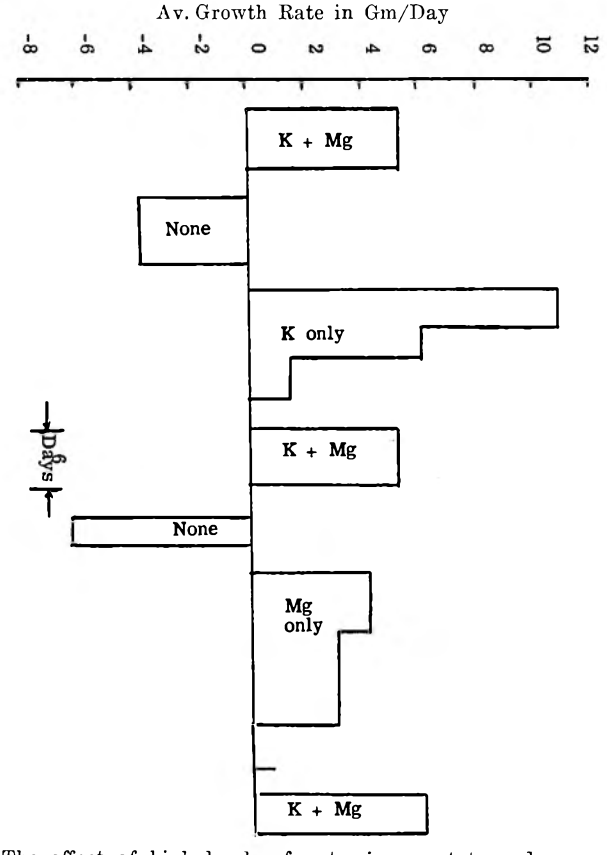

Fig. 1 The effect of high levels of potassium acetate and magnesium oxide on the growth responses of guinea pigs to amino acid supplements.

age of 11.6 and 20.0 (sucrose) and 11.8 and 8.3 (dextrin) gm/animal/day during the two periods without the extra salts.

The addition of either potassium acetate or magnesium oxide immediately reversed the losses in all groups. As can be seen from the example in figure 1, the potassium salt had

slightly more effect than the magnesium salt. However, it was only when both salts were added that a satisfactory rate of gain could be maintained. There were a few minor exceptions to the progressively decreasing response to either salt alone among the other groups, e.g., 8.0, 2.8, and 4.0 gm/day in response to magnesium oxide alone with the group receiving 30% of casein with dextrin. However, in no case was the trend completely reversed and responses of the type shown for the group receiving 25% of casein with dextrin were obtained in 90% of the cases. Also, this group contained 5 animals/group throughout the period, whereas one animal from each of the other groups had died during the first period without the extra salts.

Experiment 4, growth with different proteins. The results for the experiment in which the growth-promoting effect of different proteins was tested, are presented in table 3. In contrast to the results with younger animals, the basal group in this experiment grew at a rate of 4.5 gm/day without arginine. Since the objective of this experiment was to determine the relative values of different proteins in meeting the need for arginine, the differences that were observed were not very great. However, the substitution of gelatin for 6% of casein in the diet of the second group did permit growth that was slightly better throughout the 11-day period.

TABLE 3

Experiment 4

The effect of different proteins upon the growth rate of older guinea pigs fed purified diets [1,2]

PROTEIN	(Av. starting wt. = 717 gm)			
	24% CASEIN	18% CASEIN 6% GELATIN	18% CASEIN 10% GELATIN	24% SOYBEAN PROTEIN [3]
	gm/day	*gm/day*	*gm/day*	*gm/day*
1 week	5.1	5.7	6.4	6.9
11 days	4.5	5.5	4.8	5.6

[1] Responses listed as average gain in gm/day, 5 guinea pigs per group.

[2] All diets supplemented with 0.3% DL-methionine and 0.1% DL-tryptophan.

[3] A soybean protein obtained from the Drackett Co., Cincinnati, Ohio.

Initially there was a further improvement when the level of gelatin was increased to 10% (group 3). However, the rate of growth of this group declined during the last 4 days giving an average which was slightly less than that of the second group which received 6% of gelatin.

The 4th group which received the soybean protein grew the most rapidly during the first week. However, the growth rate of this group also decreased during the last 4 days giving an average which was about the same as that of the second group, but which was still slightly better than that of the control group.

DISCUSSION

The initial results after 4 days of amino acid supplementation were almost identical with the final results at the end of the 5 weeks of period A. This observation would indicate that it is justifiable to use short-term experiments in establishing amino acid requirements and relationships of the guinea pig under these conditions.

These longer-term experiments showed that supplementary arginine and methionine are needed by the guinea pig fed 24% casein-sucrose diets, *throughout* the rapidly-growing phase, although the need for amino acid supplements decreased with age.

The initial growth depression when 0.6% of arginine was fed to older animals was probably the first definite indication that the need for supplementary arginine had now decreased. More direct evidence of the lessening requirement for supplementary arginine was seen during the following week of the same experiment when the group receiving 0.1% of arginine grew considerably better during this week as compared to the first week. A third definite indication of a decreased requirement was seen in the experiment with different types of protein, when the group receiving the basal 24% casein-sucrose diet grew rather well without arginine.

The decreased need for supplementary methionine was shown in the second experiment also. Except for the first

week, little or no responses to supplementary methionine could be shown with guinea pigs about 4 months old. The fact that 0.3% of methionine now depressed the growth rate also suggests that the requirement had decreased.

The requirement for supplementary arginine and methionine by male guinea pigs receiving a 24% casein-sucrose diet can be estimated from these experiments. Until about the 4th month, approximately 0.5% of arginine seems to be the most effective level; from the 4th to the 5th month, 0.3% appears to be the best level; after the 5th month, little or no response can be shown to supplementary arginine. In the presence of added arginine approximately 0.3% of methionine seems to be needed for about three months; after 4 months little or no response to supplementary methionine can be shown. These figures should be regarded as only tentative and are subject to revision when more quantitative experiments are performed.

Whether the decreased need for supplementary arginine and methionine represents a true reduction in the requirements, an improved utilization of these amino acids, or a combination of thse possibilities is not known. The need for amino acids generally decreases with increasing age, due to a lower growth rate. This possibility seems to be the most likely when it is realized that the apparent decreases in the need for these amino acids approximately follow the decrease in the rate of growth of the guinea pig.

The results reported in this paper clearly indicate that extra potassium and magnesium have to be provided in order to obtain and maintain satisfactory responses to amino acid supplements. The poor responses obtained by Woolley and Sprince ('45) and Booth et al. ('49) were very likely due to their failure to include sufficient potassium and magnesium in their guinea pig diets. Although the levels of known amounts of pantothenic acid and folic acid in the diet used by Woolley and Sprince were inadequate for the guinea pig (Reid and Briggs, '54; Woodruff et al., '53) the 5% of liver powder probably made up for most, if not all, of the vitamin

deficiencies. However, the small amount of potassium and magnesium contributed by this preparation and the approximately 0.07% more potassium and magnesium from the higher salt level would still come nowhere near the 1.0% of extra potassium and 0.3% of extra magnesium that Roine et al. ('49) found necessary for the guinea pig. Even though there were only a few instances in which these older animals seemed to be adapting to the diet that was deficient in potassium and magnesium (mainly in the dextrin groups), young guinea pigs adapted to this diet from the start would probably grow about 2 to 3 gm/day as found by Woolley and Sprince ('45) and Booth et al., ('49).

Most of the immediate loss in weight observed in all 6 groups when the extra potassium and magnesium salts were removed was probably a reflection of the sensitivity of the guinea pig to dietary changes. It is very possible that the abrupt change in salt content of the ration had upset the water balance causing an immediate drop in water consumption and corresponding loss in weight. Also, the addition of either salt by itself could have immediately increased the water consumption and thereby increased the weight. Regardless of the significance of this possibility the fact remains that both salts were necessary to maintain satisfactory responses, indicating that the effect was more than just a salt and water balance effect.

The fact that both greater negative and positive effects were seen in the groups receiving 30% of casein after the omission or addition of these salts would also indicate that more than a simple water balance effect was involved. Beyond the 25% of casein level, the greater amount of phosphorous contributed by the higher levels of casein could possibly have been detrimental in the absence of extra potassium and magnesium as was recently reported by House and Hogan ('55). On the other hand, the possibility of a greater direct need for these elements for the increased protein metabolism cannot be overlooked. Recent work (Cannon et al., '51; Menaker and Kleiner, '52; Frost and Sandy, '53) has

indicated the need for extra potassium and magnesium, especially the former, in protein synthesis.

Although the soybean protein and gelatin contain about double the amount of arginine present in casein, there were only slight growth differences when the older guinea pigs were fed these different proteins. However, younger animals with their greater need for arginine, might be expected to show greater growth differences with these proteins.

SUMMARY

1. The requirements for supplementary arginine and methionine by young male guinea pigs receiving a 24% casein-sucrose diet have been shown to exist throughout the period of rapid growth.

2. The need for supplementary arginine and methionine decreased as the animals matured, the decrease approximately following the decrease in growth rate.

3. Satisfactory growth responses to amino acids were obtained only when supplementary potassium and magnesium were provided in the diet.

4. Because of the decreased need for arginine by the older animals, there were only slight differences in the growth rate when 6% of gelatin was substituted for an equivalent amount of casein and when the casein was entirely replaced with a soybean protein.

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THE INFLUENCE OF AGE AND DIET ON ASCORBIC ACID METABOLISM IN RATS [1]

DOROTHY A. EHMKE,[2] BESSIE L. DAVEY AND E. NEIGE TODHUNTER
Laboratory of Human Nutrition, School of Home Economics, University of Alabama, Tuscaloosa

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INTRODUCTION

A difference in reduced ascorbic acid content of plasma and whole blood of adult male and female rats had been observed in earlier investigations in this laboratory (Todhunter and McMillan, '46). The lower values for the females which were less than half those for the males, might have been caused by increased stores in some organs, or by higher excretion of the vitamin. The present investigation was undertaken to answer these questions, and to see whether the blood ascorbic acid level could be increased by oral feeding, or if age might be an influencing factor. Therefore, the ascorbic acid content of organs, urine, and blood of young albino rats on diets with and without additional vitamin C was determined.

METHOD

Groups of male and female albino rats of the Wistar strain were taken at weaning, maintained on three different diets and sacrificed at 63 and at 112 days of age. The diets used were as follows: (a) stock diet of laboratory checkers,[3] (b) stock diet plus 50 mg ascorbic acid daily given orally in solution, for 21 days prior to death, (c) a synthetic vitamin C-free

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[2] Present address: Medical College of Alabama, Birmingham.

[3] Ralston Purina.

diet of the following percentage composition: casein 18, Osborne and Mendel salt mixture 4, yeast 8, cod liver oil 4, vegetable oil 6, corn starch 60. This was fed for 21 days prior to death.

Urine samples were collected for 24-hour periods for three days on alternate days during the week just prior to sacrifice of the animals. Metabolism cages were constructed of fine wire mesh and were 6 inches in diameter. A raised floor of aluminum wire mesh was inserted in each cage which was then placed over a glass petri dish containing 40 ml of 6% metaphosphoric acid plus 0.4 ml of beta-hydroxyquinoline. This type of metabolism cage was specially devised so that urine fell immediately into the acid, thus avoiding any loss by oxidation or drying which might occur on the sides of the funnel-type of collection apparatus. Food was withheld during the urine collection periods, but water was provided from a fountain attached at the side of the cage. At the end of the 24-hour period the mesh floor of the cage was washed with 6% metaphosphoric acid and the total fluid was then washed into a graduated cylinder and the volume recorded. The total ascorbic acid content was determined by the method of Roe and Kuether ('43). Final readings were made in a Coleman spectrophotometer and the values were determined from a calibration curve.

When animals reached either 63 or 112 days, food was removed for 14 hours before they were anesthetized.[4] The abdominal aorta was exposed and 3 to 5 ml of blood were withdrawn in a glass syringe. The hemoglobin content of the blood was determined by the method of Sheard and Sanford ('29). Blood hematocrit was determined by the method of Roshan ('31). Total ascorbic acid content of whole blood and white cell-platelet fraction was determined by the method of Bessey, Lowry and Brock ('47) using a Beckman spectrophotometer. Reduced ascorbic acid in blood plasma was determined by the modification of the Mindlin and Butler ('38) method as described previously (Todhunter and McMillan, '46).

[4] Nembutal (0.090 mg per gram of body weight) was injected intraperitoneally.

The liver, kidneys, adrenal glands and testes or ovaries were dissected out immediately, dipped in saline solution and blotted on filter paper to remove excess fluid. To avoid change in weight during the weighing process, the separate organ from each animal was placed in a weighed, covered bottle containing enough 6% metaphosphoric acid to cover the organ. Analysis for reduced ascorbic acid in organs was by the method of Bessey ('38).

RESULTS

Analysis of variance was calculated for the data on blood, organs and urine,[5] and was used to determine significance (Snedecor, '46).

Blood data. Hemoglobin and hematocrit values for all groups were within normal limits. A sex difference in ascorbic acid content of plasma and whole blood was found in these animals, the males having approximately twice the amount of the females (table 1) which is in agreement with the findings in the previous study (Todhunter and McMillan, '46). The ascorbic acid content of the white cell-platelet fraction was significantly higher for the males than the females (table 1). The blood values for animals of both sexes receiving the synthetic diet were not significantly different from those of similar sex on the stock diet.

Thus it was found that the oral addition of ascorbic acid to the dietary intake did not produce any appreciable increase in ascorbic acid in any fraction of the blood.

The older animals did not show any significant difference in ascorbic acid content of the whole blood, plasma or white cell fraction with the exception of the females receiving the stock diet. For this group the ascorbic acid of the blood was significantly higher than at 63 days of age.

Urinary excretion of total ascorbic acid. There was no significant difference in the excretion of ascorbic acid by males

[5] Grateful acknowledgment is made to Dr. Oliver Lacey, Head, Department of Psychology, University of Alabama for assistance with the statistical analysis.

TABLE 1

Summary of blood data for rats of different age, sex and diet

AGE AND DIET	SEX	NO. OF ANIMALS	AV. WEIGHT	REDUCED ASCORBIC ACID	TOTAL ASCORBIC ACID	
				Plasma	Whole blood	White cell
			gm	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
63 Days						
Stock	M	18	180	1.25 ± 0.23 [1]	1.30 ± 0.21 [1]	14.05 ± 6.08 [1]
Stock	F	20	127	0.52 ± 0.07	0.69 ± 0.09	7.71 ± 2.35
Stock plus ascorbic acid	M	8	196	1.38 ± 0.16	1.31 ± 0.21	12.42 ± 1.22
Stock plus ascorbic acid	F	10	129	0.54 ± 0.06	0.70 ± 0.10	8.31 ± 1.83
Synthetic	M	5	174	1.29 ± 0.16	1.24 ± 0.05	10.57 ± 2.90
Synthetic	F	8	133	0.51 ± 0.07	0.65 ± 0.08	8.07 ± 1.87
112 Days						
Stock	M	17	289	1.40 ± 0.21	1.40 ± 0.22	14.46 ± 4.02
Stock	F	15	172	0.63 ± 0.14	0.90 ± 0.18	10.25 ± 2.98
Stock plus ascorbic acid	M	10	274	1.42 ± 0.34	1.55 ± 0.38	13.70 ± 2.09
Stock plus ascorbic acid	F	10	175	0.61 ± 0.10	0.80 ± 0.08	9.85 ± 2.13
Synthetic	M	7	303	1.36 ± 0.32	1.30 ± 0.15	14.78 ± 3.82
Synthetic	F	10	185	0.58 ± 0.14	0.69 ± 0.13	9.45 ± 1.80

[1] Standard deviation.

and females on the stock diet at 63 days of age (2.08 and 1.83 mg respectively) but at 112 days of age the excretion for males was higher than for females (4.09 and 1.65 mg). When calculated per 100 gm of body weight the difference between the sexes was not significant (table 2). Dumm and Ralli ('49) found an excretion of about 2 mg daily for both sexes at 30 days of age, an increase to a maximum of 3.01 ± 1.40 for males, and 4.08 ± 0.60 for females at about 45 days of age, and then a steady decrease and levelling off at about 1.40 mg at 92 days with no difference between the sexes after 65 days.

TABLE 2

Urinary excretion of total ascorbic acid
(mean of three days for all animals)

AGE AND DIET	MALES			FEMALES		
	Ascorbic acid			Ascorbic acid		
	per 24 hr.	per 100 gm body weight		per 24 hr.	per 100 gm body weight	
	mg	*mg*	*S.D.*	*mg*	*mg*	*S.D.*
63 Days						
Stock	2.08	1.06	0.59	1.83	1.43	0.65
Stock, plus ascorbic acid	5.50	2.91	1.02	5.91	4.66	1.43
Synthetic	1.42	0.81	0.53	1.44	1.11	0.49
112 Days						
Stock	4.09	1.41	0.73	1.65	0.96	0.48
Stock, plus ascorbic acid	6.70	2.55	0.95	4.13	2.37	0.84
Synthetic	3.87	1.28	0.75	1.60	0.87	0.27

The addition of 50 mg of ascorbic acid daily to the diet caused a significant increase in the excretion of ascorbic acid. However, the increase in excretion was small compared to the amount of ascorbic acid fed to the animals, indicating destruction within the body, or storage within some tissue not analyzed.

Liver. The liver weight was higher in males than in females and increased with age. Males on the stock diet at 63 days of age had an average liver weight of 5.951 ± 0.62 gm, and at 112 days 8.131 ± 1.37 gm; for females liver weights for the respective age groups were 4.654 ± 0.45 and 5.214 ± 0.52. On a

TABLE 3

Reduced ascorbic acid content of organs

AGE AND DIET	SEX	LIVER	KIDNEY	ADRENALS	TESTES OR OVARY
		mg/gm	*mg/gm*	*mg/gm*	*mg/gm*
63 Days					
Stock	M	0.25 ± 0.086 [1]	0.11 ± 0.045 [1]	2.44 ± 0.986 [1]	0.27 ± 0.092 [1]
Stock	F	0.21 ± 0.065	0.06 ± 0.033	3.36 ± 0.811	0.35 ± 0.088
Stock plus ascorbic acid	M	0.31 ± 0.150	0.10 ± 0.026	3.43 ± 0.796	0.26 ± 0.014
Stock plus ascorbic acid	F	0.23 ± 0.024	0.06 ± 0.050	3.37 ± 0.708	0.36 ± 0.082
Synthetic	M	0.26 ± 0.020	0.14 ± 0.044	3.86 ± 1.155	0.21 ± 0.012
Synthetic	F	0.23 ± 0.032	0.04 ± 0.018	2.51 ± 0.476	0.24 ± 0.028
112 Days					
Stock	M	0.23 ± 0.044	0.10 ± 0.032	2.96 ± 1.474	0.25 ± 0.019
Stock	F	0.22 ± 0.035	0.04 ± 0.021	2.91 ± 0.629	0.34 ± 0.120
Stock plus ascorbic acid	M	0.22 ± 0.021	0.11 ± 0.017	3.29 ± 0.895	0.25 ± 0.022
Stock plus ascorbic acid	F	0.22 ± 0.042	0.06 ± 0.029	2.79 ± 0.938	0.39 ± 0.202
Synthetic	M	0.22 ± 0.081	0.07 ± 0.048	1.98 ± 0.704	0.21 ± 0.013
Synthetic	F	0.20 ± 0.041	0.03 ± 0.023	2.55 ± 1.019	0.28 ± 0.149

[1] Standard deviation.

unit weight basis (table 3) there was no significant difference between sexes in liver ascorbic acid. Morehouse and Guerrant ('51), have reported that at 6 weeks of age males had a higher ascorbic acid content of the liver which could not be attributed to difference in food intake. Svirbely ('36) also found a lower content for females. The ascorbic acid content of the liver of males given by both these investigators was similar to that reported here.

The feeding of ascorbic acid increased the liver content of the vitamin in males at 63 days of age; Samuels ('48), using males only, obtained an increase in liver and kidney content when 50 mg of ascorbic acid were given orally each day for 5 weeks.

Adrenals. The females had larger adrenals, 43.0 $\pm$ 6.3 mg and 55.3 $\pm$ 1.1 mg than males, 33.3 $\pm$ 6.8 and 40.3 $\pm$ 8.4, at both 63 and 112 days of age, although there was considerable difference in the total body weight of the animals. The mean body weight for all males at 63 days was 183 gm and at 112 days it was 284 gm; for females the corresponding weights were 155 and 174 gm. On a unit weight basis (table 3) the females showed no significant differences from the males in the ascorbic acid content of adrenals. Neither age nor dietary ascorbic acid intake influenced the adrenal ascorbic acid content.

Kidneys. The kidneys of males at 63 days of age averaged 1.664 $\pm$ 21 and 2.289 $\pm$ 0.34 gm at 112 days, whereas the values for females were 1.263 $\pm$ 0.12 and 1.442 $\pm$ 0.18 gm at the same age. Thus, the kidneys were found to increase in weight with increasing age and those of the males were significantly higher in weight and in ascorbic acid on a unit weight basis. The addition of dietary ascorbic acid did not increase the vitamin content of the kidney (table 3).

Sex organs. Compared on the basis of milligrams of ascorbic acid per gram of organ the ovaries contained significantly larger amounts of ascorbic acid than did the testes.

Neither age nor diet caused any significant change in ascorbic acid in testes of the males, or in ovaries of the females (table 3).

DISCUSSION

A lower content of reduced ascorbic acid in the plasma and whole blood of young rats of 63 and 112 days of age was found for the females and was of the same order of difference as in the earlier study on mature animals (Todhunter and McMillan, '46). However, the amounts present in the 112-day-old animals were 1.40 mg per 100 ml of plasma for males and 0.63 mg for females on the stock ration (table 1), and 1.25 and 0.52 mg respectively for the 63-day-old animals; for the mature animals of the earlier study (Todhunter and McMillan, '46) the values were 0.87 and 0.33 mg for the respective sexes. The white cell-platelet fraction showed similar lower values for the females. Morehouse and Guerrant ('52) have reported that plasma ascorbic acid decreases with age.

Oral feeding of 50 mg of ascorbic acid per day gave no significant increase in blood levels for the females and it must therefore be assumed that the blood levels of ascorbic acid in females are adequate for body metabolism. This assumption is further verified by the fact that the excretion of ascorbic acid by the females was comparable to that of the males, indicating that there was no greater destruction or utilization within the body.

The reduced ascorbic acid content of the organs of the females was not significantly different from that of the males, except in the case of the kidneys where the females had significantly less ascorbic acid per unit of weight. Also, the kidneys were smaller in the female. The kidney content of ascorbic acid for the males is within the range reported by other workers (Samuels, '48; Sure et al., '39) but these investigators used males only or did not report the sexes. Females had larger adrenal glands than males and while the ascorbic acid content was the same per gram of adrenal this would mean a higher total amount present in these glands of the female. It is not likely that this would account for the significantly

lower blood levels in the females and this latter finding is still unexplained.

SUMMARY

Groups of male and female rats received ascorbic acid-deficient diets, and diets supplemented with ascorbic acid. The animals were sacrificed at 63 and 112 days; plasma, whole blood and white cell-platelets, liver, kidney, adrenals, ovaries and testes and urine were analyzed for ascorbic acid content.

Females in both age groups had significantly lower content of ascorbic acid in plasma, whole blood and platelets in both age groups, and a significantly lower content in the kidneys for both age groups.

Oral supplements of 50 mg daily of ascorbic acid for 21 days prior to death caused no increase in ascorbic acid content of blood or organs for either sex. Urinary excretion of ascorbic acid was increased for both sexes.

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ASCORBIC ACID UTILIZATION BY WOMEN

RESPONSE OF BLOOD SERUM AND WHITE BLOOD CELLS TO INCREASING LEVELS OF INTAKE [1]

ELLEN H. MORSE, MARTHA POTGIETER
AND GEORGIANNA R. WALKER
School of Home Economics and Storrs Agricultural Experiment Station, University of Connecticut, Storrs [2]

ONE FIGURE

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In a recent study Potgieter et al. ('55) reported the response of the ascorbic acid levels of blood serum and of night urinary excretion to increasing levels of intake of the vitamin by 20 women. The average ascorbic acid level in the serum reached its maximum of 1.49 mg per 100 ml on a daily intake of 95 mg of the vitamin. Further increase in intake of ascorbic acid did not increase the serum level.

The present study concerns the relationship between the ascorbic acid level in the blood serum and that in the white blood cells of a second group of women on the same graded levels of ascorbic acid supplementation as in the previous study.

Discussions of white cell ascorbic acid and serum ascorbic acid suggest that the white cells may be a better indicator of tissue saturation than blood serum. A review by Lowry ('52) sums up the information on the ascorbic acid content of white cells based on his own work and that of other in-

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vestigators. Since then studies of experimental ascorbic acid depletion of humans have shown that white cell ascorbic acid dropped somewhat less rapidly than serum ascorbic acid (Davey et al., '52; Steele et al., '52). Feeding 800 mg of ascorbic acid daily for 4 days to 10 human subjects, following a vitamin C depletion period, resulted in a prompt rise in serum values to an average of 2.3 mg and in white cells to an average of 33.4 mg (Steele et al., '52). In 13 human subjects (Steele et al., '53), on a diet containing 10 mg of ascorbic acid a day, white cell and serum ascorbic acid both dropped to low levels. When the ascorbic acid intake was gradually increased to 40 mg a day, the white cell level rose significantly while the serum value remained at the low level of 0.2 mg per 100 ml.

In the present study, levels of vitamin C intake were chosen which fall within the range of intake that has been found in population groups in this country (Tucker et al., '52). The effect of these various controlled levels of intake on serum and white cell ascorbic acid was studied.

PROCEDURE

Subjects. Nineteen women, 27 to 64 years of age, patients at a state training school and hospital for the handicapped [3] served as subjects during the winter and spring of 1953–54. All were physically normal and in good health, as shown by physical examination. The subjects were examined at the beginning and end of the study for clinical signs of vitamin C deficiency. No noticeable changes that might be related

[3] Mansfield State Training School and Hospital, Mansfield Depot, Connecticut. Thanks are extended to the medical and dietary staffs of the school as follows: To Dr. Gail F. Moxon, M.D., resident doctor, for the physical examinations; to Dr. Joseph E. Nowrey, M.D., resident doctor, and his assistants for taking the venous blood samples; to Dr. Luke Grotano, D.D.S., resident dentist, for the dental examinations; to Mrs. Pauline Duckett, chief dietition, and to the dietary staff of the women's dining room, for cooperation in the collection of dietary data and food samples; to the 19 mentally retarded women who served so cheerfully and cooperatively as subjects; and to Dr. Neil A. Dayton, M.D., Superintendent of the Training School, for making the institution available for the study and for his continued interest and encouragement in research work.

to changes in vitamin C intake were noted during the period of the study.

Dietary ascorbic acid. Throughout the 5 months of the study the subjects were on the regular institution diet except that all high vitamin C foods such as citrus fruits, tomatoes, pineapple, and raw cabbage were omitted. This was done in order to provide a somewhat restricted and relatively constant dietary level of ascorbic acid. Other fruits and vegetables were always served to the subjects in place of those omitted.

The food intake of the subjects was recorded by the authors on 16 days chosen at random near the beginning and again on 16 days near the end of the 5-month period. Food consumption was recorded for each subject in terms of the number of servings, or fraction of serving, of each food. Servings were weighed at intervals to determine average weight. Samples of foods were collected in the dining hall from time to time, and were analyzed for total ascorbic acid as in the preceding study (Potgieter et al., '55), using an adaptation of the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43). The ascorbic acid intake of individual subjects, for each of the 32 days on which food intake was recorded, was calculated using the food values obtained by analysis.

Ascorbic acid supplementation. After 7 weeks on the diet restricted in ascorbic acid, vitamin supplementation was begun. Each subject received a 25-mg tablet of ascorbic acid daily for 5 weeks. This was followed by a 50-mg daily supplement for 5 weeks, then by a 100-mg supplement each day for the final 5-week period.

Serum and white cell ascorbic acid determination. Serum and white cell ascorbic acid determinations were made on venous blood samples about one week after the beginning of the study, at the end of the period on the restricted diet without vitamin C supplementation, and at the end of each 5-week period on the three levels of supplementation. The blood samples were always taken at 10:00 A.M., three to 4 hours after a vitamin C-free breakfast. Preparation and analysis of the serum were carried out according to the pro-

cedure outlined in the Northeast Regional Publication on Techniques ('51).

The blood samples for white cell determination were prepared in quadruplicate and the determinations made by the method of Bessey (Gyorgy, '50).

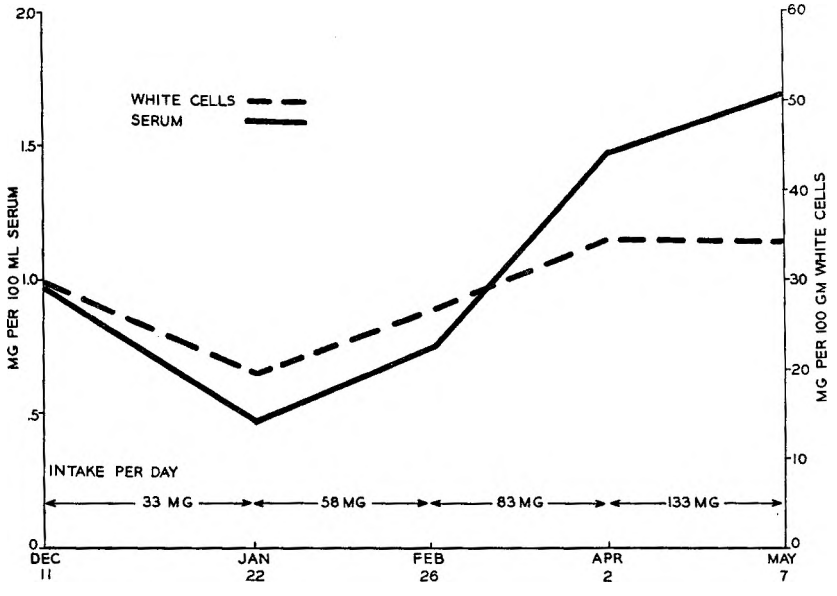

Fig. 1 Average values for ascorbic acid in blood serum and in white blood cells for 19 women receiving an average dietary intake of 33 mg ascorbic acid per day, with the following ascorbic acid supplementation: 25 mg from Jan. 22 to Feb. 26, 50 mg from Feb. 26 to Apr. 2, 100 mg from Apr. 2 to May 7.

RESULTS

Ascorbic acid intake. The average daily ascorbic acid intake from food during the 32 days under observation was 33 mg, with individual average daily intakes for the 19 women ranging from 24 to 44 mg. This average daily value, plus the vitamin supplementation at the three levels, brought the average total daily ascorbic acid intake for the three supplemental periods to 58, 83, and 133 mg respectively.

Serum ascorbic acid levels. Serum ascorbic acid levels at the beginning of the study (one week after the restricted

diet was begun) averaged 0.97 mg per 100 ml of serum. At the end of the 7-week period on an average daily intake of 33 mg, the average serum value for the group had dropped to 0.47 ± 0.033 mg (see fig. 1). After the 5 weeks on a daily intake of 58 mg, the average serum ascorbic acid level was 0.74 ± 0.061 mg. During the period on a daily intake of 83 mg, the serum levels continued to rise, reaching an average of 1.47 ± 0.077 mg. At the end of the period on an intake of 133 mg per day, the average serum value was 1.69 ± 0.062 mg.

White blood cell ascorbic acid levels. The average white cell ascorbic acid level for the group at the beginning of the study was 29.94 mg per 100 gm of white cells. After the period of restricted vitamin C intake, the average level fell to 19.55 ±0.929 mg (see fig. 1). As a result of the 5 weeks on the 25-mg supplement, the average level rose to 26.67 ± 0.693 mg. Following the 50-mg supplement, the average level was 34.55 ± 1.058 mg. There was very little change in the white cell ascorbic acid level after the period on the 100-mg supplement. The average value was 34.15 ± 1.098 mg.

DISCUSSION

In the present study, the degree of fluctuation of white cell ascorbic acid was less than that of serum ascorbic acid. The former dropped 35% from the time of the first blood sample to the end of the period on the 33-mg intake. During the same time the serum level dropped 52%.

During the period on the 58-mg intake, the white cell level rose 36%. With the intake of 83 mg, the rate of increase was similar, namely 30%. During these two periods the serum level increased first 57%, then an additional 99%. At the end of the 5-week period on a daily intake of 83 mg the white cell concentration had evidently reached its maximum level (34.55 mg) since it did not rise further on the 133-mg intake. In the case of the subjects studied by Steele et al. ('52) the white cell level reached about the same value (33.4 mg) after

4 days on an intake of 800 mg per day. There was a slight but significant rise of 15% in the serum level as a result of the 133-mg level of intake (significant at the 0.002 level on a two-tail paired test).

In all 19 of the subjects, both serum and white cell ascorbic acid levels rose on the 58-mg intake, and made an additional rise on the 83-mg intake. In 17 of the subjects, the serum ascorbic acid reached a level of over 1.0 mg on the 83-mg intake; three of these reached this level while on the 58-mg daily intake. In two subjects the serum ascorbic acid did not reach the 1.0-mg level until on the 133-mg level of intake. On the 133-mg intake, 15 of the subjects showed further increase in serum ascorbic acid, while 4 showed a decrease. However, on this last level of intake the average white cell ascorbic acid remained about the same, with 8 subjects showing an increase and 11 a decrease.

Statistical analysis of the data showed that the coefficients of correlation between the serum levels and the white cell levels of ascorbic acid were as follows: on the 33-mg intake, $r = 0.63$ and is significant at the 1% level; on the 58-mg intake, $r = 0.46$ and is significant at the 5% level; on the 83-mg intake, $r = 0.55$ and is again significant at the 5% level; on the 133 mg intake, $r = 0.11$ and is not significant. The regression of white cell level, Y, on serum level, X, is $Y = 16.9 + 10.8X$ for the 76 items. Analysis of variance showed there was no curvature.

The optimal level of ascorbic acid intake is still a question. It is generally agreed that an intake just adequate to prevent scurvy is not enough to maintain saturation levels in body tissues and fluids. It is assumed that tissue saturation is an advantage at times of infection or injury. That level of intake which will maintain saturation of body tissues is here regarded as the optimal daily intake. An optimal nutritional status with respect to vitamin C might be considered that level of ascorbic acid nutriture which maintains tissue satu-

ration, in contrast to an "adequate" nutritional status which does not produce saturation but which prevents signs of deficiency.

The results reported here suggest that under the conditions of the present study, with intakes ranging from 33 to 83 mg per day, the serum level was a reasonably good indicator of the degree of white cell saturation. The method for determining serum ascorbic acid is a shorter and simpler procedure than that for white cell ascorbic acid. In clinical studies and nutritional surveys of population groups, under less carefully controlled conditions, the serum ascorbic acid is generally accepted as an indicator of general nutritional status, but not of the degree of tissue saturation.

SUMMARY

The average blood serum ascorbic acid level in 19 women subjects fell from 0.97 mg to 0.47 mg per 100 ml of serum during a period of 6 weeks on a diet containing an average of 33 mg of ascorbic acid per day. The average serum level increased to 0.74 mg following 5 weeks on an intake of 58 mg, to 1.47 mg following 5 weeks on an intake of 83 mg, and to 1.69 mg following an equal period on 133 mg.

Fluctuations in white blood cell levels were consistent with those in the serum though somewhat less marked. During the period on the restricted intake, the average white blood cell level for the subjects fell from 29.94 mg to 19.55 mg ascorbic acid per 100 gm of white cells. Following the first two periods of supplementation, the white cell level rose to 26.67 mg and 34.55 mg respectively. There was no further increase in white cell ascorbic acid level as a result of increasing the intake to 133 mg per day.

Statistical analysis of the data indicates that, with intakes between 33 and 83 mg per day, under the conditions of the present study, the average serum level was a reasonably good indicator of the average ascorbic acid content of the white cells.

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