CCXXXII. STANDARDIZATION OF THE RAT FOR BIO-ASSAY OF VITAMIN E¹

BY K. E. MASON AND W. L. BRYAN

*From the Department of Anatomy, Vanderbilt University
School of Medicine, Nashville, Tennessee*

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A SURVEY of numerous studies concerned with the experimental production of vitamin E deficiency reveals a rather wide variation in the time necessary to induce sterility in rats of either sex. In male rats reared from weaning on vitamin E-deficient diets, the time of experimental feeding necessary to produce degenerative changes in the germinal epithelium may vary from 50 days or less [Mason, 1926; Ringsted, 1936] to 90–150 days [Evans & Burr, 1927; Kudrjaschov, 1930; Juhász-Schäffer, 1931]. It has also been the experience of many investigators that a variable proportion of female rats reared on vitamin E-deficient diets will complete one or more pregnancies before exhibiting a resorption-gestation typical of vitamin E depletion. This variable occurrence of initial fertility has proved particularly annoying and time-consuming in the preparation of rats for the bio-assay of vitamin E, and has necessitated the production of a proven resorption in all rats prior to their use in assay tests. Attempts to eliminate the occurrence of this phenomenon by the use of slightly rancid fat in the hopes of destroying possible traces of vitamin E in the experimental diets used have not proved particularly effective. Furthermore, such a procedure seems highly undesirable in view of the observations [Kudrjaschov, 1935] that products of a ketone nature formed in rancid fats are capable of preventing implantation in rats and may even cause resorption of the foetus if implantation is successful. Proper limitation of the vitamin E content of the diet supplied to mothers whose offspring are to be selected for vitamin E deficiency studies appears to be a much more successful and acceptable method for elimination of initial fertility [Ringsted, 1936], but its general applicability is limited by the care and expense involved in the preparation of stock diets containing a uniformly low content of vitamin E. More recently, Bacharach *et al.* [1937] report the occurrence of resorption-gestations in the first pregnancies of 96 out of 104 vitamin E-deficient rats examined prior to 1937, and in 100 % of 316 rats tested since that time, but fail to mention the extent to which the lactating young had access to the maternal diet or the composition of the latter.

Although it has been rather generally accepted that the vitamin E reserves of young rats and the variable response of the latter to vitamin E depletion are significantly influenced by the vitamin E content of the maternal diet, no effort has been made to evaluate the mechanisms concerned. The present communication deals with an attempt to determine to what extent the vitamin E reserves of newly weaned rats are due to direct consumption of the maternal diet as the young adopt free-eating habits during the latter part of lactation, and to what

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extent they can be attributed to placental and mammary transfer. These studies have also demonstrated the reliability of a relatively simple procedure which completely eliminates the occurrence of initial fertility and greatly facilitates the preparation and standardization of rats to be used in the bio-assay of vitamin E, or for experimental studies relating to vitamin E deficiency.

Vitamin E storage in offspring given access to maternal diet

In order to determine the storage capacity for vitamin E in suckling rats, the offspring of mothers maintained on diets containing variable amounts of vitamin E were permitted access to the maternal diet during the period of lactation, after which they were reared upon a vitamin E-deficient diet (diet 68, Table II). The data summarized in Table I demonstrate conclusively that delayed

Table I. *Onset of sterility in vitamin E-deficient rats in relation to the vitamin E content of the diet available during lactation*

Group	No. of rats	Diet to which rats had access before vitamin E depletion was begun	Age when put on vitamin E-deficient diet days	Days on vitamin E-deficient diet before onset of sterility†		
				Min.	Max.	Average
A	24	Stock diet,* supplemented with a special suckling diet containing 20% ether-extracted wheat germ still containing appreciable amounts of vitamin E	21-30	160	324	240
B	10		25-29	150	200	180
C	13	Stock diet, supplemented with fresh lettuce 2-3 times weekly	21	90	218	151‡
D	18		21	95	160	130
E	10	Stock diet only	21	58	113	82§
F	5		21	80	100	90
G	178	Vitamin E content of maternal diet unrestricted and often excessive, but mother and offspring given vitamin E-deficient diet after 10th day of lactation	21	32	71	43.4
H	118		21	30	70	48.4

* The stock diet used in these studies was a commercial dog biscuit which affords a daily intake of from 2-3 times the minimal requirement of vitamin E for the rat.

† Onset of sterility in female rats was taken as the beginning of the first gestation period during which resorption occurred. In males this was estimated on the basis of histological examination of the testis and epididymis and can be regarded as only approximate.

‡ Two rats resorbed during first gestation, 5 completed one, 3 completed two and 3 completed three gestations before resorption occurred.

§ Resorption during first gestation occurred in 8 out of 10 rats.

|| First litter fertility completely eliminated.

onset of sterility in rats of both sexes is roughly proportional to the vitamin E content of the maternal diet, when the young have access to the latter throughout the period of lactation. The particularly long depletion period in the rats of groups A and B is due partly to the higher content of vitamin E in the diet, as compared with the other groups, and partly to maintenance on the maternal diet beyond the 21st day of life (at which time the rats in our colony are as a matter of routine weighed, removed from the mother and placed upon the experimental diets). Attempts to hasten the onset of sterility in a number of animals in groups A to D by aeration of the lard at 100° for 10 hr. before its inclusion in the diet were unsuccessful. Even the unsupplemented stock diet conferred an appreciable storage in suckling rats permitted to consume it prior to the 21st day of life (groups E and F). However, when the offspring were denied access to the maternal diet and consumed only a vitamin E-deficient diet from the

ERRATA

Volume XXXII, No. 10

for col. 2 of Table I *read:*

No. of
rats

24 ♀

10 ♂

13 ♀

18 ♂

10 ♀

5 ♂

178 ♀

118 ♂

page 1787, Table II. *after* Wheat germ oil *read* ‡

assumption of free-eating habits, sterility supervened very early (groups G and H). The latter two groups represent a summary of results obtained in the experiments discussed in the following sections.

Vitamin E storage in offspring denied access to the maternal diet

Three groups of 13 rats each, containing an equal distribution of litter-mate sisters, were selected for breeding and reared upon the diets S, S + E and D + E, respectively (Table II). It was estimated that the S diet supplied approximately 2–3 times the minimal requirement of vitamin E for the rat, as determined by bio-assay of known quantities of the diet and measurements of the daily food consumption. It was estimated that the S + E diet supplied approximately

Table II. *Composition of diets*

Diet S		Diet S + E	
Stock diet (commercial dog biscuit)		Stock diet	80
		Fresh wheat germ	20
Diet D + E		Diet 68	
Casein*	22	Casein*	22
Cornstarch	54	Cornstarch	54
Lard	20	Lard	20
Salts†	4	Salts	4
Brewer's yeast	1 g. daily	To each 100 g. of this diet, 8 g. brewer's yeast and 2 g. cod liver oil were added at the time of feeding	
Cod liver oil	6 drops daily		
Wheat germ oil	6 „		

* Finely granulated commercial casein found to be suitable for use in vitamin E deficient diets without further purification.

† Modification of the Osborne-Mendel salt mixture suggested by Wesson [1932].

‡ Kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minn.

15 times, and the D + E diet about 10 times, the minimal daily requirement of vitamin E. The rats were all maintained individually in wire cages with screen floors throughout the experiment. The reproductive performance was essentially the same in all three groups. Litters were limited to a maximum of six young. Six mothers whose lactation record was decidedly inferior were discarded. 90% of the 93 litters delivered by the remaining rats were successfully weaned, yielding 666 offspring with an average body weight of 42.5 g. at the 21st day of life. This is well above the minimum standard of 36 g. maintained in this laboratory for 21-day-old rats selected for studies relating to vitamin deficiency and reproductive performance. We have found that rats failing to meet this standard are decidedly inferior and frequently give very atypical results.

Throughout each lactation period the rats of the S and S + E groups were given diet 68 (Table II) and, to avoid too abrupt a dietary change, were also supplied with the regular diet until its removal on the 10th day of lactation. Those of the D + E group were deprived of the wheat germ oil supplement between the 10th and 21st days of lactation. By this means, the offspring of all three groups were denied any source of vitamin E except that obtained through placental transfer and through the mammary secretion.

It is of particular interest that the paralysis characteristic of the suckling young of mothers low in vitamin E [Evans & Burr, 1928] appeared in 20% of litters reared by mothers of the S group, but in none of the offspring of the S + E and D + E groups, indicating a significantly greater storage of vitamin E in the latter. It has since been found that this phenomenon rarely appears

when the vitamin E-deficient diet is substituted for the S diet at the 14th–15th days of lactation. We have failed to find in the paralysed rats, and in a large series of rats known to have been severely depleted of vitamin E from birth to adult life, the thyroid hypoplasia noted in such animals by Singer [1936] and Barrie [1937]; this confirms a recent report by Telford *et al.* [1938].

(a) *Placental and mammary transfer to female offspring.* Three groups of 92, 32 and 36 female rats selected respectively from the S, S + E and D + E mothers, were reared upon diet 68 and mated with normal males after they had attained body weights of approximately 150 g. Many began their pregnancies between the 30th and 40th days of experiment and, despite the occurrence of pseudo-pregnancy in 12 % of rats, all were successfully inseminated after an average of 45 days (i.e. at 66 days of age). In all 160 rats the first gestation terminated in complete resorption. Graphic representation of the average body growth of each group throughout the gestation-resorption indicated that the resorptive process was more severe in the offspring from mothers on the S diet than in those from mothers on the S + E and D + E diets, which is in accord with the small protection afforded to the testes of male offspring from the latter two groups compared with those from mothers on the S diet, as discussed below. Although we have failed to note any significant difference in the responses of offspring from mothers on the three types of breeding diets when used for the bio-assay of vitamin E concentrates, the data upon which strictly comparative estimates can be made are limited to 10 rats of each group and are considered insufficient to warrant a more positive statement.

(b) *Placental and mammary transfer to male offspring.* Three groups of 35, 21 and 21 male rats selected respectively from the S, S + E and D + E mothers, averaging 44.6 g. in body weight at the 21st day, were reared upon diet 68. A total of 95 testes and corresponding epididymides obtained either by operative removal or at autopsy after various intervals of feeding were weighed, prepared for histological study and classified according to the predominance of one or more of the five stages into which the degenerative process has been arbitrarily divided [Mason, 1926]. Special reference should be made to the value of histological examination of the epididymis in confirming and augmenting conclusions drawn regarding the previous and current alterations in the germinal epithelium of the testis. The results of this analysis are summarized in Table III.

It will be noted that in the offspring of mothers fed on the S diet (series S, Table III) early evidence of testicular injury appeared after as little as 30 days of feeding. At this time (51 days of age) mature spermatozoa were usually making their first appearance in the ducts of the caput epididymis both in the vitamin E-deficient rats and in litter-mate brothers reared from weaning upon the stock diet. Although the rats of this series had been receiving restricted amounts of vitamin E from birth, and some had spontaneously recovered from mild degrees of paralysis apparent during the last few days of lactation, the prepubertal growth and maturation of the testes exhibited no significant inhibition. Further investigations are being made in regard to the latter question, and to the earliest period at which the irreparable nature of the testicular injury can be demonstrated. The onset of testicular injury was approximately 10 days earlier than that observed in the earlier studies of Mason [1926] and from 5 to 10 days earlier than that noted by Ringsted [1936] in rats reared by mothers on a breeding diet containing a low but adequate content of vitamin E and subsequently fed on a vitamin E-deficient diet. The testicular damage observed in testes obtained after the same interval of feeding, and in the different seminiferous tubules of any one testis, was unusually uniform, especially when compared with the

Table III. *Extent to which placental and mammary transfer of vitamin E affects the onset of testicular degeneration in male rats reared on a vitamin E-deficient diet*

No. days on exp. after weaning	Series S		Series S + E		Series D + E	
	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration
30	5	N?-1	—	—	—	—
40	3	N?-1	—	—	—	—
	9	1	1	N?	1	N?
	6	1-2	—	—	—	—
50	1	1-2	—	—	3	N
	5	2-3-4	7	N-N?	3	N?-1
	5	3-4-5	2	N?-1	2	1-2
60	—	—	6	N?-1	3	N?-1
	1	3-4-5	3	1-2	3	1-2
	4	4-5	3	2-3-4	3	2-3-4
70	—	—	2	2-3-4	2	3-4-5
	—	—	4	3-4-5	4	4-5
80	—	—	2	4-5	2	4-5
Total no. of testes	39		30		26	

picture presented in testes of rats maintained for prolonged periods on vitamin E-deficient diets before the onset of sterility.

In the offspring of mothers fed on the S + E and D + E diets (series S + E and D + E, Table III) testicular degeneration appeared approximately 20 days later than in the S series described above, demonstrating an appreciable placental and mammary transfer of vitamin E when the daily intake of the mother was increased to 10 to 15 times the minimal. Preliminary attempts to measure the extent of this transfer, by feeding different amounts of a carefully assayed concentrate of vitamin E to 21-day-old rats treated in the same manner as those of the S series, indicated that the smallest amount of vitamin E necessary to delay the onset of testicular degeneration 20 days was approximately twice the single curative dose necessary for preventing resorption in female rats.

(c) *Placental versus mammary transfer.* The results of another series of experiments, which indicate that the mammary gland is almost entirely responsible for the transfer of vitamin E to the offspring, warrant brief mention. Three rats were given approximately 200 times the minimal single curative dose of vitamin E¹ during their period of gestation. Immediately after delivery, and before the young had opportunity to suckle, the litters were exchanged with those of recently delivered mothers fed on the stock diet. One other rat was given approximately 400 times the minimal single curative dose distributed over a period extending from the 13th day of pregnancy to the 7th day of lactation. The 22 male and 18 female offspring without access to any dietary source of E during lactation were reared upon the vitamin E-deficient diet.

In six male offspring whose storage of vitamin E was limited to that received during intra-uterine life, the time of onset of testicular degeneration was essentially the same as observed in the rats of series S (Table III). In 12 males suckled by mothers whose storage of the excess vitamin E administered during gestation apparently permitted close to the maximum mammary transfer of vitamin E to the adopted young, the testicular injury appeared about 20 days later, as noted in the rats of series S + E and D + E (Table III). Prolonged feeding of the

¹ In the form of a concentrate prepared from wheat germ oil by molecular distillation; kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minnesota.

vitamin E concentrate during lactation was deemed inadvisable because of possible contamination of the young with traces of the vitamin from the body or food of the mother. In the one instance where excess of vitamin E was given during late gestation and the first week of lactation, the four male offspring exhibited an additional delay of about 10 days in the onset of testicular degeneration, when compared with the latter group. It is particularly noteworthy that the 18 female rats derived from the seven litters just discussed, successfully impregnated at an average age of 64 days, invariably resorbed during their first gestation.

It is hoped that studies now in progress using a greater excess of vitamin E and a much larger series of animals will afford a more adequate measure of the maximum and relative efficiencies of the placental and mammary barriers in the transfer of vitamin E. The preliminary observations reported above merely suggest that the vitamin E storage of the offspring of mothers fed on the S + E and D + E diets represents close to the maximum attainable through combined placental and mammary transfer, that this storage is almost entirely due to mammary transmission of the vitamin and that increasing the daily vitamin E intake of the pregnant and lactating rats to several hundred times the minimal requirement does not permit enough vitamin E to pass the placental and mammary barriers to prevent resorption during the first gestation in female offspring otherwise deprived of vitamin E.

DISCUSSION

The results presented in this report are in close accord with other observations regarding the limited placental and mammary transfer of vitamin A [Dann, 1932; 1934; Baumann *et al.* 1934] and of vitamin D [McCollum *et al.* 1927] in the rat. Questions regarding storage capacity and excretion after administration of an excess of the fat-soluble vitamins, the "physiological" selectivity of the placental and mammary barriers and possible differences in storage capacity in the offspring during intra-uterine and postnatal life, are beyond the scope of the present discussion but offer interesting fields for further exploration.

The relative ineffectiveness of placental and mammary transfer of vitamin E in the rat, even when the maternal intake is exceedingly high, affords conclusive evidence that voluntary consumption of the stock diet by the young rat during the latter part of lactation is the primary factor responsible for the irregularity and delay frequently noted in the time of onset of sterility in vitamin E-deficient rats of both sexes. The complete elimination of initial fertility in female vitamin E-deficient rats through the simple expedient of substituting a vitamin E-deficient diet before the middle of lactation, with the production of offspring whose vitamin E reserves are very low from the time of birth and little influenced by minor variations in the vitamin E content of the maternal diet (which may be selected from the standpoint of cheapness and excellence for breeding purposes), should greatly facilitate the standardization of rats for the bio-assay of vitamin E and permit a much more accurate evaluation of assays conducted in different laboratories.

Furthermore, the established reliability of the first gestation period for assay tests (when properly controlled by the use of untreated litter-mate controls), which can be begun after an average of 45 days from the time of weaning, together with the economy of space and diet due to the smaller size of the test animals, are distinct advantages. The virginal state of the reproductive tract prior to the gestation used for the bio-assay test also appears appreciably to increase the

number of successful impregnations after positive matings [Bacharach *et al.* 1937] and should impart greater reliability to the assay procedure. We have obtained exceedingly uniform and satisfactory results in a series of more than 180 bio-assay tests conducted on rats prepared in the manner described.

In the present study, the male proved to be a more delicate indicator of vitamin E storage in the young rat than did the female. Certain problems relative to sex differences in storage capacity and vitamin requirement during pre-pubertal and post-pubertal life are being more thoroughly investigated in order better to evaluate the possible usefulness of the male rat for vitamin E assays.

SUMMARY

The variable responses of young rats of both sexes to vitamin E depletion can be closely correlated with the vitamin E content of the maternal diet to which the young have access after the adoption of free-eating habits during the latter part of lactation. By substitution of a vitamin E-deficient diet for the maternal diet during the last half of lactation, to limit the source of vitamin E to that obtained through the placenta and mammary gland, the initial storage of the vitamin in newly weaned offspring is decidedly limited and but little influenced by the vitamin E content of the maternal diet.

When the maternal diet contains but several times the minimal requirement of vitamin E, the vitamin E reserves of the offspring are negligible and, when reared upon vitamin E-deficient diets, males exhibit the onset of testicular degeneration between the 50th and 60th days of life, coincidently with the first appearance of spermatozoa. Increasing the vitamin E content of the maternal diet to about 15–20 times the minimal delays the onset of testicular injury about 20 days, which represents about the maximum protection obtainable through combined placental and mammary transfer, as indicated by preliminary tests in which the maternal intake was increased several hundredfold. Most of the vitamin transfer is accomplished through the mammary secretion.

Female rats whose initial vitamin E storage is limited to that obtained through placental and mammary transfer invariably resorb during their first gestation period when reared on vitamin E-deficient diets, even when the maternal intake is increased several hundredfold. The value of these observations in the improvement and standardization of methods for the bio-assay of vitamin E is emphasized.

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