CCLXXXI. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

I. THE INFLUENCE OF VITAMIN E DEFICIENCY ON IMPLANTATION

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No assay for vitamin E is possible in the absence of an international or other accepted unit and a standard preparation to be used in a test contemporaneous with that on the test substance. Comparative evaluations, however, as distinct from assays, can be carried out between any two sources of vitamin E tested simultaneously, in so far as we have knowledge of the relationship between dose and response and of the variation in response shown by the animals used in the test. Very little information is to be found in the literature on either point, though a recent paper of Palmer [1937] contains some interesting relevant observations.

The response of vitamin E-deficient animals to the vitamin, being of the "all or nothing" type, has to be treated as a quantum, since there is no possibility of measuring or assessing gradation in that response. In other words, it is necessary to take groups of adequate size as the animal testing unit, and to determine the percentage of positive responses given by all the members of each group.

Attempts to standardize this kind of technique were begun in these laboratories nearly 18 months ago, and our experience since then has led to some modifications which may increase the possibility of quantitative comparative tests. In this paper, it is proposed to discuss certain conditions that are, in our opinion, essential to any quantitative test, and to record some observations upon a phenomenon that has hitherto apparently escaped notice.

A basal diet for vitamin E tests must satisfy the two criteria of a suitable vitamin-deficient diet. First, it must be completely deficient in vitamin E; secondly, it must not be quantitatively or qualitatively lacking in any other food constituent necessary for the production of living young, since failure to give birth to living young is taken as proof of the absence of vitamin E and the production of living young as proof of its presence.

The diet used in these laboratories is based upon that of Evans and associates, as given by Rowlands & Singer [1936], with the slight modification that we have used a simplified salt mixture (identical with that used in our vitamin A-deficient diets). Further, since the number of animals receiving this diet in our laboratories is sufficiently large to necessitate mixing fresh supplies daily, the cod liver oil is incorporated in the diet, instead of being fed as a separate supplement to each animal. Young does of the London strain of Wistar albino rat are placed upon this diet at weaning. As soon as they are sexually mature

and exhibit oestrus, as shown by the usual examination of vaginal smears, they are mated with bucks of proved fertility.

If, at weaning, animals receiving this diet also receive a daily supplement of some material containing adequate supplies of vitamin E, a high percentage of full-term litters is produced. Table I shows the results obtained when five

Table I. *Effect of diet Rees 1. Unsupplemented or supplemented, on live litter rate*

Supplement, from day of weaning	No. of implanted animals	No. of live litters	Live litter rate (%)
None	422	8	2*
Lettuce, c. 2 g. daily	25	25	100
Wheat germ oil I, 112 mg. daily	15	13	87
Wheat germ oil II, 112 mg. daily	6	5†	84
Liquid unsaponifiable fraction from wheat germ oil, 21 mg. daily	21	20	95
Arachis oil, 112 mg. daily	10	3	30
Olive oil, 112 mg. daily	9	0	0

* Since January 1937, 316 animals on unsupplemented diet have given no live litters.

† One animal had a second resorption-gestation in spite of continued dosage.

different materials were fed prophylactically with the basal diet. Of these, it is clear that the levels of the first three provided at least optimum amounts of vitamin E, while those of the other two did not. Nevertheless, the results indicate clearly the presence of vitamin E in the sample of arachis oil. Sure [1926] found indications that vitamin E was present in pea-nut oil, but this does not appear to have received clear confirmation before. Our results also show a complete absence of vitamin E from the cod liver oil used in our diet, thereby also confirming Sure's finding [1927]. We now invariably use the same cod liver oil as diluent for oils and concentrates submitted to vitamin E test as supplements to the diet.

In the absence of prophylactic treatment with vitamin E, typical gestation-resorptions occur. Records of 420 animals show the production of only eight litters, and these were born more than 12 months ago in circumstances that did not rule out the possibility of some accidental administration of vitamin E. Over 300 animals have received the unsupplemented diet since that time, and none has produced a litter after mating with a buck of proved fertility.

It has always been the practice, as far as one can gather from published work, to use animals of this kind, that is, animals that have undergone at least one gestation-resorption, in order to make certain that they have been completely depleted of their vitamin E reserves. Such animals will be referred to as R.P. (resorbed primiparae).

It occurred to us that some time might be saved by omitting altogether the process of submitting animals to one resorption on the diet alone and administering the dose of test substance to animals immediately after their first mating, since there is a probability approaching certainty that without vitamin E they would undergo a gestation-resorption. We therefore introduced this practice some months ago. Naturally, a certain number of animals, used as negative controls, are reared upon the diet alone; these invariably undergo a gestation-resorption after a successful implantation.

This change in experimental technique has revealed an entirely unexpected phenomenon, and one that not only has practical value as an improvement in the technique of testing for vitamin E, but may also have physiological implications of considerable importance.

When the doe rat in oestrus is brought into contact with an adult fertile buck, the mating that follows may not always be "positive", as evidenced by the presence either of the vaginal plug or of sperm in the vagina, or of both. Animals failing to show positive mating are remated until success is obtained, or rejected altogether for experimental purposes.

In a healthy stock of animals a very high percentage of positive matings, though not always of positive first matings, is recorded. From the 12th to the 16th day after such mating, and generally between the 13th and 15th days, there should appear in the vaginal contents a certain amount of blood, easily detectable with the naked eye. This is known variously as the placental sign, the erythrocyte sign, the implantation sign, and indicates that fertilized ova have been implanted on the uterine wall. Unless implantation occurs, the animal must be remated at the next oestrus. When implantation does not occur, if the animal has been receiving doses of a test substance, these doses will have been wasted—a matter of considerable annoyance when, as sometimes happens, the test substance is only available in very small quantities as the result of weeks of laborious chemical work. Moreover, the time of the operator also is wasted in feeding and examining animals incapable of indicating the presence or absence of vitamin E. This failure to implant is well known to workers in sex physiology, but its consequences are more irksome in vitamin E investigations than in many others.

Tests for vitamin E can, then, only be completed with animals that have shown both positive mating and implantation. We observed, after a number of virgin vitamin E-deficient animals had been positively mated, that a higher proportion of these animals than of R.P. animals seemed to show implantation. The matter appeared worth careful investigation, and we have therefore kept records not only of the numbers of litters born to animals showing the implantation sign, but also of the number of positively mated animals showing implantation. The marked difference between the implantation rates of R.P. and virgin animals is shown in Table II.

Table II. *Implantation rates*

Type of animal	No. of animals showing positive mating	No. of implantations	Implantation rate %
R.P.	286	174	61
Virgin	143	138	96

It became necessary next to examine whether this difference was correlated with the administration of vitamin E to the experimental animals. We therefore divided both the R.P. and the virgin animals into two groups, according as the percentage of live litters born to successfully implanted animals was lower or greater than 10; those showing less than one living litter for every ten successful implantations are held to have received no dose of vitamin E and those showing more to have received a dose. The records in Table II are reclassified in this way in Table III.

Table III. *Implantation rates*

Type of animal	Activity of dose given	No. of animals showing positive mating	No. of implantations	Implantation rate %
R.P.	+	173	108	62
R.P.	0	113	68	60
Virgin	+	64	61	95
Virgin	0	79	77	97

It seems clear that the addition of vitamin E to the diet of either R.P. or virgin mated animals has no significant effect on implantation rates and that the difference between the rates must be due to some other cause.

We are able to reject the possibility that a lower implantation rate is characteristic of reproduction in all animals that have already had one litter. No information on this subject has been found during a rather brief survey of the literature, but Wiesner [1937] states that his experience has been exactly the opposite, and that this is what would be expected on general physiological grounds.

There are two other possible explanations of the phenomenon recorded. R.P. animals, when used for test purposes, will naturally have undergone a longer period of vitamin E deficiency than virgin animals, for the time taken from their first mating through a gestation resorption to the second mating and administration of the test substance may be anything from 4 to 6 weeks. It is conceivable that the extra period of deprivation produces effects that express themselves in an increased difficulty of implantation. This explanation appears to us to have a *prima facie* improbability. The deficient animals have, at the time of their first mating, been fed on the deficient diet for 2 months or more; that they have been "run out" of vitamin E is shown by a resorption rate not significantly less than 100%. Yet the rate of implantation of these animals after positive mating is also not significantly less than 100%. It seems unlikely that a further period of 5 or 6 weeks on the same diet would result in a sudden 40% lowering of the implantation rate, especially as the animals seem perfectly healthy and normal in all respects save reproductive power.

The only other explanation that we can suggest is that the actual process of undergoing a gestation-resorption itself affects the reproductive processes in the doe rat in such a way as to increase the difficulty of implantation. Investigations are in hand in an attempt to obtain further evidence on this question. It may, however, be emphasized that, whatever the true explanation, some surprising conclusions must be drawn from the established facts.

It has generally been held that the effect of vitamin E deficiency on the female rat, as distinct from that on the male rat, is completely "reversible"; that is to say, it only affects the particular litter *in utero*, and has no effect on subsequent litters, provided that vitamin E is restored to the animal's diet. Only one finding in conflict with this view appears in the literature; Juhasz-Schäffer [1931] reports that the size of a litter produced by a cured vitamin E-deficient rat bears an inverse relation to the number of previous gestation-resorptions. The number of animals used by Juhasz-Schäffer was, however, not very great; further, he has apparently not taken adequately into account the fact that the size of litter tends to fall from the third or fourth onward, even on a normal diet.

The results reported here make it difficult to resist the conclusion that a period of vitamin E deficiency, from weaning to sexual maturity, has an effect on the maternal organism much more deep-seated than has hitherto been believed. Since the percentage of implantations is very high in virgin vitamin E-deficient does, this deep-seated consequence of vitamin E deficiency apparently only affects implantation after the rat has undergone a gestation-resorption.

It seems that the process of resorption itself brings about changes in the reproductive apparatus of the female rat, which express themselves as a lowered fertilizability of the ova or as a lowered probability for those fertilized ova to become implanted embryos. In view of the findings of Rowlands & Singer

[1936] and of Barrie [1937, 1, 2] as to the changes in endocrine organs demonstrable in vitamin E-deficient rats, we need, perhaps, not be surprised that changes are also to be found, even if only so far by indirect methods, in the reproductive system, which is fundamentally affected by endocrine action.

SUMMARY

The various fates that may attend the female rat, reared from weaning to maturity on a vitamin E-free diet and then given material under test for the presence of vitamin E are shown in Fig. 1.

Fig. 1. *Reproductive behaviour of female rat on vitamin E-deficient diet.*

* Also some other rare conditions, about which little appears to be known.

Failed matings (A) do not appear to be more frequent in the absence than in the presence of the vitamin, but no detailed figures on this point are available from our records. R.P. and virgin animals behave similarly.

Failed ovulation (B) is rare, whether with R.P. or with virgin animals, both with and without the vitamin.

Failed implantation, or failed fertilization ($C_1 + C_2$), seems independent of the presence or absence of the vitamin, but R.P. animals show a much higher percentage of failures than do virgin animals. C_1 is the ordinary pseudo-pregnancy: C_2 is an abnormality which appears seldom, if ever, to have been recorded. Though we are unable to state precisely whether C_2 actually predominates over C_1 , we have certainly found that it occurs frequently under our experimental conditions.

E is the simple gestation-resorption by which alone vitamin E-deficiency has hitherto been properly recognized.

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