CLXXIII. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

II. FURTHER OBSERVATIONS ON VITAMIN E DEFICIENCY AND IMPLANTATION

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THE response to vitamin E treatment cannot be graded satisfactorily. Attempts to make use of the size of a litter, while ignoring the viability of its individual pups, would seem to have little justification; nor can the observer always be certain that he has in fact observed all the pups, dead or alive, that have actually been expelled from the maternal organism. The simplest procedure seems, therefore, to treat the response as of the all or nothing kind, and it becomes necessary to define what we mean by the birth of a litter.

We assess as a positive response the appearance of any number of animals, from one upwards and of whatever viability, whether they are found alive or not; it is clearly impossible to know in every instance whether all members of a litter were actually breathing at the time of birth.

We were at one time faced with a further difficulty. On the 22nd or 23rd day after mating it would sometimes be found impossible to tell whether a resorption had occurred and the response was accordingly to be judged negative, or whether a small number of non-viable young had indeed been born, only to be eaten by the mother before the cage had been inspected. In the absence of any sign of young, such a response, which should by definition have been judged positive, would have been recorded as negative. Accordingly, all cages containing does that have shown positive implantation are now fitted, on the 19th or 20th day after mating, with a movable grid, of mesh about $\frac{3}{4}$ in., placed $2\frac{1}{2}$ in. above the cage bottom. The doe remains on this grid, but the young that are born fall through on to the cage bottom. They seldom survive, but it is in any event our practice to destroy them; our sole concern is to have objective evidence that they have come into existence. Even so, a small number of animals may be consumed by the mothers before they have ever been deposited on the cage bottom (or grid); no account can be taken of these, whether the grid is used or not. The phrase "expelled from the maternal organism" must be read as meaning completely expelled and actually deposited by the mother on the floor of the cage.

We take as a measure of response what we call the fertility rate, or the percentage fertility, that is, the number of litters, as defined above, in relation to the number of positive implantations expressed as a percentage. The use of the fertility rate is exemplified later in this paper.

In an earlier publication [1937] we have advocated the use of virgin rats as test animals for vitamin E. Apart from the time saved by avoiding a preliminary gestation-resorption, a further economy is achieved in both time and material, as virgin animals show a higher implantation rate than those (R.P. animals) that have already undergone a gestation-resorption.

These results have been confirmed on a further large number of animals, drawn from our colony of highly inbred Wistar (London Strain) albino rats. The results are shown in Table I. In Table II we have combined the results from animals discussed in our earlier communication and those upon which Table I is based, but have re-divided them into 3 groups, according as the implanted animals produced no litters, up to one litter for every five implantations or more than this number. It will be seen from Table II that, at all 3 levels of vitamin E dosage, virgin animals showed much higher implantation rates than did the R.P. animals; the figures also confirm our former observation that the percentage of implantations is not affected by the level of vitamin E dosage after positive mating.

Table I. *Implantation rates*

Type of animal	No. of animals showing positive mating	No. of implantations	Implantation rate (%)
R.P.	59	32	54
Virgin	542	469	87

Table II. *Implantation rates*

	R.P. animals	Virgin animals
Dosage 0 (no litters)	61% (115)	90% (221)
Dosage $\pm$ (to 20% fertility rate)	74% (42)	90% (147)
Dosage + (>20% fertility rate)	56% (188)	87% (317)
Totals	59.7% (345)	88.6% (685)

The figures in parentheses indicate the numbers of animals mated.

In our previous paper we suggested as the most probable cause for the reduced implantation rate of R.P. animals that the process of gestation-resorption itself produces some permanent change in the animal's reproductive system. In order to test this point further, the following experiment has been carried out.

Twenty-five pairs of young female rats were placed at weaning on our basal diet, REES 1. When the animals came into oestrus, one of each pair was mated with a buck of known fertility, and mating was repeated if necessary until all 25 animals had shown the "plug" or the presence of live spermatozoa in the vaginal contents. Of these 25 animals, 24 showed the placental sign on the following 15th or 16th day; the 25th animal was re-mated and then became implanted. None of these 25 animals produced any living young, and all showed the symptoms of typical gestation-resorptions. They were all re-mated on showing oestrus again after the normal gestation period, and were then also given for 5 days a daily dose of 4.5 mg. of an extract prepared from wheat germ oil. Of these 25 R.P. animals, 15 showed failed implantation, giving the low implantation rate of 40%. Of the 10 implanted animals 5 had live litters, a "fertility rate" of 50%.

When the R.P. animals were about to be mated for the second time, their sisters were examined and also mated at the first normal oestrus. These were therefore virgin animals that had been submitted to the deficient diet REES 1 for some 3-4 weeks longer than is usual with our test animals, while they were waiting for their litter-mate sisters to be re-mated; their period on the diet, however, was identical with that of these R.P. litter-sisters. Of the 25 virgin animals, 21 showed positive implantations; this implantation rate of 84% is not far below the average for the whole of our recorded virgin animals. All 25 animals received the same dose of wheat germ oil extract as their R.P. sisters,

Table III. *Litter-mate comparisons*

Type of animal	Mated	Implanted	Fertile	Implantation rate %	Fertility rate %
R.P.	25	10	5	40	50
Virgin	25	21	16	84	76

and 16 of the 21 animals with positive implantations produced live litters, a fertility rate of 76 %. The results are summarized in Table III.

It seems certain that in groups of 25 animals the difference between 40 % implantation for R.P. animals, and 84 % implantation for virgin animals, is highly significant; the differences in implantation rates cannot be due to different periods of vitamin E deficiency, since both groups of animals received the diet for the same time. It is probable, though not certain, that the difference in fertility rates of the 10 R.P. animals and the 21 virgin animals is also significant, indicating that the R.P. animals have not only a lower rate of implantation, compared with virgin animals, but also a higher vitamin E threshold. The relatively small number of R.P. animals involved in this comparison, however, makes it impossible for this point to be conclusively demonstrated.

We have some contributory evidence for this apparent difference in threshold. A sample of carefully preserved wheat germ oil concentrate (unsaponifiable matter largely freed from sterols by freezing) was in use for the preparation of a standard dose-response curve in experiments that are being made the subject of another communication. When tested on R.P. animals, this concentrate gave the following results:

Dose mg.	Implanted	Live litters	Fertility rate %
105	10	6	60
230	9	8	89

For the preparation of the dose-response curve only virgin animals were used. The comparable figures for fertility rates were:

Dose mg.	Implanted	Live litters	Fertility rate %
22.5	39	34	87.2
11.25	44	15	34.1

For both the higher and the lower doses of these two groups, it appears that R.P. animals may require 6 times as much vitamin E as virgin animals, if they are to show the same fertility rate. This threshold ratio is the highest we have encountered: it is more usually in the neighbourhood of 4 or 5 to 1.

In the classical monograph of Evans & Burr [1927] occur some observations on the relation between vitamin E and implantation. I am much indebted to Prof. J. C. Drummond for calling my attention to these remarks. Evans & Burr wrote: "We must call attention, firstly, to the fact that between five and eighteen per cent of all cases of 'positive' matings in normal animals, i.e. those held upon natural foods, result in failure to implant. Secondly, animals maintained on a basal ration and used to demonstrate the incidence of the specific sterility disease should not show an appreciably higher proportion of failed implantations than do animals maintained upon natural foods¹. In our records of somewhat over fifteen hundred positive matings in animals on natural foods, only 82 to 95 per cent are followed by implantations. A much lower implantation per cent denotes either peculiar food inadequacy different in nature from that due to low E, or denotes the presence of some special toxic factor or general constitutional debility²." The footnotes ¹ and ² above also deserve quotation in full. "¹ The reader may naturally inquire as to whether

failure to implant may not possibly represent a more advanced stage of the particular sterility disease due to lack of vitamin E. It would appear that a conclusively negative answer can be given here inasmuch as, although we now have evidence of the gradual depletion of the body in its residual stores of E and the consequent necessity in old animals of somewhat higher curative doses of wheat germ oil, for instance, than is essential in young ones on the same diet, we do not have evidence of added difficulty in implantation in such cases. Furthermore, and more decisive—many cases of persistent failed implantation were given wheat germ oil in excess daily for over a month without effect on implantation.” “² We would especially inform the reader in some groups of animals reared and held on the basal ration, the ovulation, acceptance of coition and especially the implantation have been so deficient as to demand the rejection of most individuals of the group. It is only when these phenomena are normal that animals can be used for experiments calculated to show the presence or absence of vitamin E in any dietary regime.” The authors then go on to discuss the nature of food deficiencies and special toxic factors.

Whatever effect such factors may have on implantation rates, they have been as far as possible excluded in our experiments, for both groups of animals received treatments identical except in the one variable under examination. It is not clear whether the quoted remarks of Evans & Burr apply only to virgin animals or not; if they do, their observations are consistent with our own.

One possibility must be mentioned, namely, that it is the occurrence of gestation as such, and not the pathological process of a gestation-resorption, to which is to be attributed the reduced implantation rate. This, as we mentioned in our earlier publication [1937], is contrary to experience. For example, a very large colony of animals of the same strain as our own stock was maintained by the Macaulay Laboratory at the Institute of Animal Genetics in Edinburgh University for some years. Detailed records of mating are available for a considerable number of those animals [Wiesner, 1938]. They indicate that animals under 150 days old, mated for the first time, had a fertility rate of about 89 %. The animals were receiving a normal stock diet, and no question of vitamin E deficiency therefore arises: any failure in fertility of animals that had undergone positive mating must have been almost entirely due to failed implantation. The fertility of animals of similar age, positively mated after the birth of a litter, also showed about 89 % fertility, indicating no change in implantation rate.

In spite of this evidence, we have planned a further crucial experiment to distinguish between the effects on implantation rate of a “normal” gestation and the pathological condition of gestation-resorption. Meanwhile, we feel more than ever inclined to the view that toxic by-products from the resorbed embryos have inflicted some lasting damage on the uterine or other mechanism of implantation.

REFERENCES

- Bacharach, Allchorne & Glynn (1937). *Biochem. J.* **31**, 2287.
Evans & Burr (1927). *Mem. Univ. Calif.* No. 8.
Wiesner (1938). Private communication.