CCCXXVI. THE DETERMINATION OF VITAMIN A BY MEANS OF ITS INFLUENCE ON THE VAGINAL CONTENTS OF THE RAT.

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EVANS AND BISHOP [1922] first showed that when young rats were fed on a diet deficient in vitamin A, the cyclic changes in the vagina were apparently upset, for "cornified cells" (keratinised cells) were found continuously day after day in the vagina. If vitamin A was given the condition was cleared up and the regular cycles became easily recognised. Evans and Burr [1928] showed that the oestrous cycles occurred naturally during the period when keratinised cells appeared continuously but the signs of the cycles, as usually given by the vagina, were hidden.

Other workers have confirmed the findings of Evans and Bishop, and Baumann and Steenbock [1932] showed that this evidence of vitamin A deficiency responded to treatment with daily doses of carotene in Wesson oil (hardened cottonseed oil). They suggested that this symptom of vitamin A deficiency and its cure by the giving of vitamin A might be made the basis of a method for the quantitative estimation of vitamin A.

The suggestion was very attractive to us as this response of the animal appeared to be specific for vitamin A. (Methods based on the growth response of animals are open to many well-known criticisms.) It also seemed possible that this method might offer a means of estimating the vitamin A potency of a preparation suspected of being labile, for it might be possible to obtain measurable results from giving one dose only of the preparation to each rat suitably prepared. This has proved to be possible. A logarithmic curve has been obtained relating (a) the number of days elapsing before cornified cells disappeared from the vagina, and (b) the size of the dose given. The duration of the cure obtained by giving vitamin A also bears a curvilinear relationship to the dose given, but this curve is not logarithmic.

METHOD.

(a) *General scheme of the test.* The only information available as to the sensitivity of the vagina to doses of vitamin A was given by Baumann and Steenbock [1932] who had found that 10 γ carotene per day restored dioestrous smears within a week. Klussmann and Simola [1933] found that daily doses of 1-5 γ carotene caused the cornified cells to disappear. None of these workers stated exactly what condition the rats were in when doses were first given. We therefore had to determine exactly what criteria we would use (a) to consider a rat ready for the test and (b) to consider a rat to have responded to a dose of vitamin A. It is, of course, unnecessary for workers in different laboratories to adopt the same criteria for biological reactions provided that a standard of reference is available

in terms of which an estimation can be made, and provided also that the workers in different laboratories have shown that they can obtain, by means of their own technique, a graded response to graded doses of the purest form available of the active substance.

To consider a rat ready for the test, we decided that it must have had cornified cells (almost exclusively) in the vaginal smear daily for 10 days. As the rats' reserves of vitamin A become exhausted, these cells appear suddenly in very large numbers and with only slight admixture with leucocytes or none at all.

To consider a rat to have responded to a dose of vitamin A, we decided that cornified cells should be completely absent from the smear or present in very small numbers, not more than a stray group of perhaps half a dozen in the whole smear taken.

(b) *The preparation of the rats.* The vaginal contents of each rat which had attained a weight of 130 g. on a diet containing only moderate amounts of vitamin A were examined daily by Evans's simple method of removing a little of the material from the vagina with a small spatula and examining it at once in a drop of tap water under the low power (2/3) of a microscope. By the time that it was ascertained that cycles were occurring normally the rat had usually attained a weight of 140 g. It was then given the vitamin A-free diet in general use in this laboratory until the vaginal smear had contained cornified cells for 10 consecutive days. The rat was then considered ready for the test.

(c) *Dosing of the rats.* As the rats became ready for the experiment they were distributed among 7 groups of 10–12 rats per group. All the rats of any one group were given the same dose of cod-liver oil. On the day when they were considered ready the rats of the different groups were given in a single dose on one occasion 5, 10, 20, 40, 60, 100 and 200 mg. respectively of an average sample of cod-liver oil. The same vitamin A-free diet was continued, and the vaginal contents were examined daily. When the smear contained no cornified cells or only perhaps half a dozen of those cells, the rat was considered to have responded to the dose of cod-liver oil. The number of days elapsing between giving the dose and getting the response was considered the result. One rat in the whole series responded the day after dosing; that was estimated as 1. When a rat's smears contained cornified cells for 2 days after dosing, and was free from them on the third day, the response was estimated as 3. The longest interval noted between dosing and response was 10 days. Some of the rats given the lower doses of cod-liver oil did not respond at all though smears were taken for as many as 20 days after dosing. Some of them died without giving any response. For the purpose of averaging results the figure 10 was therefore given to each rat that did not respond within 10 days.

The curve of response.

The averages of the results from the animals in the different groups were taken as the responses to the different doses of cod-liver oil (Table I). When plotted against the respective doses the results gave a curve in which only the two lowest doses were somewhat irregularly placed (Fig. 1). That the curve is logarithmic may be seen from Fig. 2 in which the average responses of the groups are plotted against the logarithms of the doses of cod-liver oil given. The best straight line through these points was calculated as $y = 13.2 - 4.5x$. Thus the curve relating the average responses of the groups of rats to the dose of vitamin A given is represented by the equation $y = 13.2 - 4.5 \log x$.

It is interesting to note that this curve of response is logarithmic although it is in no way a growth curve.

Table I. *Response of rats to a single dose of cod-liver oil shown by changes in the vaginal contents.*

Dose of cod-liver oil (Z). mg.	No. of rats on test	Interval between first dose and first appearance of leucocytes. Days	Interval between first day of cure and day when ready for second test, i.e. duration of cure.* Days	No. of rats which reached this stage
5	10	9.3	11.0	3
10	9	10.0	—	0
20	10	7.0	16.8	6
40	10	5.7	18.6	7
60	10	5.3	19.8	9
100	10	4.0	24.7	9
200	12	2.9	45.0	9

* The interval between the first day of cure and the day when ready for second test includes (a) the time during which normal cycles were occurring and (b) the 10 successive days of cornified cells in the vagina which were regarded as the indication that the rat was ready for a second test.

Fig. 1. Curve of response relating the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

Fig. 2. Curve of response relating the logarithm of the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

Duration of cure from a single dose of cod-liver oil.

Vaginal smears of the rats which had been used for obtaining the curve of response already described were still taken daily to determine when each rat might be considered ready for another test; that was when a rat had again had cornified cells in the vagina for 10 successive days. For this measurement the rats which had not responded to the first dose within 10 days were, of course, ignored. The rats that had responded to the lower doses of cod-liver oil had at least one cycle before cornified cells again appeared continuously in the vagina. The rats that had received the largest dose of cod-liver oil had 6 or 7 cycles before cornified cells appeared continuously and obliterated them.

The time taken by a rat to become ready for a second test was reckoned as the interval between the first day of its cure from the first dose and the day when it had again shown cornified cells for 10 successive days. The averages of the times taken by the rats in the different groups were plotted against the respective doses of cod-liver oil given. If the 10 days of continuous cornification (regarded as the indication that the rat was ready for a second test) are subtracted from each of these averages, the remainder may be regarded as the duration of the cures. The response is plotted in Fig. 3. The curve is not logarithmic. It is quite clear that

Fig. 3. Curve of response relating the dose of vitamin A to the "duration of the cure" of the condition of the vaginal contents.

not only does the size of dose influence the rapidity of the response, but that it also influences the duration of the rat's resistance to a subsequent shortage of vitamin A.

The possibility of using the same rats for several tests.

Rats which had responded to a dose of cod-liver oil once were, when again ready, given a second dose of cod-liver oil equal to the first; the responses were measured in the same way. There were fewer rats available for the second test than for the first because several rats given the lower doses in the first test had not responded and were therefore not comparable with those which had responded and been again depleted. In each group one or two rats showed greater sensitivity in the second test than in the first, but most were apparently less sensitive, and it was necessary to compare averages. For the purpose of these

comparisons therefore, averages of the first test results were recalculated from those rats only which were used in the second test (Table II).

Table II. *Comparison between first and second tests of the same doses of cod-liver oil given to the same rats.*

The figures in this table were compiled from those rats only which survived for the second test.

Dose of cod-liver oil (Z). mg.	No. of rats given a second dose	Time required for response		Duration of response		No. of rats that survived the 2nd test
		In 1st test. Days	In 2nd test. Days	In 1st test. Days	In 2nd test. Days	
5	3	8.0	No response	11.0	—	—
10	0	—	—	—	—	—
20	6	5.0	7.7	10.5	16.5	2
40	7	3.9	8.3	17.5	14.0	2
60	9	4.8	8.2	18.0	17.3	3
100	9*(8)	4.0	5.4	25.2	18.5	6
200	9	3.2	4.0	45.0	40.7	9

* One of these died 3 days after receiving the dose.

On the whole the rats took a longer time to respond to the second dose of cod-liver oil than to the first, and in the groups containing a reasonable number of animals, the duration of cure following the second dose was shorter than the cure following the first. Another indication of the lessened sensitivity of the rats is the fact that of the 42 rats which responded within 10 days to the first dose, 20 did not respond within 10 days to the second dose. Thus under the conditions adopted for this test, estimations of vitamin A activity would apparently have to be made entirely on rats used thus for the first time or entirely on rats used thus for the second time. There should be no indiscriminate use of any rats which happened to be ready when needed. It is possible, however, that some modification of the test (such as the adoption of 5 successive days of cornification instead of 10 as the criterion for considering a rat ready for a test) might prevent the lessening of the sensitivity of the rats, but there is no evidence in these results to indicate that it would do so.

As there were only 22 survivors of the second test (rats which completed 10 successive days of cornification after their second cure), no third test was attempted.

Weights of rats during the test.

Only 6 rats out of 62 lost as much as 10 g. in weight during the period of feeding on the vitamin A-deficient diet, up to the end of the first 10 days when cornified cells appeared daily in the vagina. This is, therefore, an earlier symptom of vitamin A deficiency in adult rats than loss of weight, and is similar to Evans's experience with young growing rats. Of the 22 rats which survived the second duration test, 4 lost at least 10 g. in weight before their second period of 10 successive days of cornification was completed.

Accuracy of the test.

As a response which varies in magnitude and which can be measured gives a more accurate result than a simple "all or none" response, no calculations were based on the number of rats which were or were not cured within a given time.

The two groups of rats given the lowest doses of cod-liver oil could not be used for estimating the accuracy of the test, as in one of them, 7, and in the other 10 did not respond at all. Using the figure 10, the maximum and only value assigned to these, would therefore give a false idea of the accuracy of the

result, as in one group there would be no variation at all, and very little in the second. In the three groups given the next highest doses, there were 4, 3 and 1 rats respectively that did not respond. These 8 rats obviously cannot be excluded from the calculation, but their inclusion at the value of 10 each will bring a lower value for the standard deviation than the true value for the test. The total number of rats from which the calculation could then be made was lamentably small, *viz.* 52, distributed in five groups. The value of σ obtained from these is 2.86, whence the probable error of a result obtained with 10 rats is +36.2 or -26.4% which is slightly greater than that obtained in a 3 weeks' growth test on young female rats in the same laboratory.

It must, however, be recognised that this figure is obtained from a calculation with a very small number of rats and, moreover, there may be other ways of measuring the effects that would give more accurate results.

SUMMARY.

Changes in the vaginal contents of the rat brought about by withholding vitamin A from the diet, and the restoration to the normal condition brought about by the giving of vitamin A, have been made the basis for a quantitative method of estimation of vitamin A.

The occurrence of keratinised cells for 10 successive days in the vagina was taken as an indication of the exhaustion of the rat's reserves of vitamin A. The disappearance of these cells from the vagina was taken as an indication of "cure". These criteria have been applied to the estimation of the vitamin A content of one dose only of the vitamin A-containing substance, that dose being given on the tenth day of the period of continuous cornified cells in the vagina.

The relation between the dose given and the mean number of days elapsing between the giving of the dose and the disappearance of cornified cells from the vagina is curvilinear. The curve is logarithmic in shape.

The duration of the cure effected by one dose of vitamin A, *i.e.* the time elapsing between the day when the cornified cells disappeared and the day when cornified cells had again appeared continuously for 10 successive days, also bears a curvilinear relation to the dose of vitamin A given, but this curve is not logarithmic.

The accuracy of this method of estimation appears to be somewhat less than that obtained by the criterion, increase in weight in 3 weeks of female rats which have been given daily doses of vitamin A after they had ceased to grow on a diet deficient in that factor.

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