III. INVESTIGATIONS INTO VITAMIN A-FREE BASAL DIETS. II.

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THE experiments described in this paper were carried out with a two-fold object. It was desired first to discover what effect deprivation of vitamin A had on the lymphatic tissue in various parts of the body, and secondly to observe simultaneously whether the difference in growth-promoting properties of a complete diet and of a vitamin A-free diet supplemented with vitamin A, already described in a previous communication [Bacharach, 1933], could be confirmed and correlated with the reserves of vitamin A in the livers of the experimental animals. The results of the observations on the lymphatic tissues will not be reported here in full (see note on p. 21).

Eighteen animals, nine of each sex, were used in this experiment, and their arrangement is shown in Table I. One group of six animals was given the full stock diet (S) described in the earlier paper (p. 6). This diet was also the diet upon which the parents of all the experimental animals had been fed during the whole of their adolescence and maturity, and upon which the experimental animals in groups 2 and 3 had been fed up to beginning the feeding of the vitamin A-free diet.

The second group was given the vitamin A-free diet (C) described in the same paper (p. 14). To this diet was added a daily dose of the unsaponifiable matter from fish-liver oil dissolved in arachis oil; the amount of this dose and the method of expressing it are discussed below. No irradiated ergosterol was fed to these animals.

The third group of animals received diet C, but no supplementary source of vitamin A.

The experiment was continued for 150 days, at the end of which all the animals were killed. Their livers were tested for vitamin A in the manner described below, and various other portions of tissues and organs were set on one side for histological examination (see note on p. 21).

In the absence of any unit of vitamin A as yet in universal use (the international standard solution of carotene having not been long enough in circulation for the necessary biological determinations to be made) it is proposed to express the activity of the concentrate fed to the animals, and the vitamin content of their livers, in terms of a standard cod-liver oil, compared colorimetrically by the antimony trichloride test. The oil adopted as a standard has a Carr-Price value of (approximately) 10, and a value of 16 when this is determined by the method of Smith and Hazley [1930]. The latter value has been taken for purposes of comparison in this paper. All figures for extract and livers given below are in terms of equivalent milligrams of such an oil.

It may be added that such an oil has a content of 10β per g., in the unit of Smith and Hazley, gives 900 B.U. per g., calculated by the method of Moore [1930] and has a value of $E_{1\text{ cm}}^{1\%} 328m\mu = 0.7$.

The vitamin A content of the extract fed to the animals in group 2 was determined in the manner described by Smith and Hazley [1930] and was that of 50 mg., of the defined cod-liver oil. The livers were treated with dilute alkali and the extract for the antimony trichloride test was made exactly as described by Moore [1930].

Fig. 1 shows the average growth curves of the animals in all three groups. The three litters were respectively 20, 21 and 24 days old at the time of the beginning of the experiment, and there is little doubt that the relatively high weight at which the animals in group 3 showed signs of ceasing to grow, and the relatively long time that it took them to reach a growth plateau, are associated with the length of time elapsing before they were put on to the deficient diet [cf. Nelson, 1928].

Although, for obvious reasons, no direct measure was possible of the vitamin A reserves of the animals at the beginning of the experiment, an approximate measure was obtained by taking 12 young animals entirely at random from the general stock of which the experimental animals had formed part, and carrying out a colorimetric determination of the vitamin A content of their pooled livers. This proved to be 100 mg. C.L.O. per liver. The animals all weighed approximately 40 g. and were between 4 and 5 weeks old. They were weaned and had been eating diet S for themselves for a week or so. It is clear that the experimental animals began to receive the three diets at a time when their vitamin A reserves, at any rate in the liver, were definitely low compared with the undoubtedly high reserves of vitamin A that must have been present in the livers of their mothers, as evidenced by the livers of those experimental animals, described in Table I, which received the stock diet S throughout the experimental period.

The young experimental animals used in this investigation had received, directly or indirectly, diet S from birth until they were about 4 weeks old;

Fig. 1. Average growth curves for three groups of three males and three groups of three females on stock, supplemented basal, and unsupplemented basal vitamin A-free diets for experimental period of 150 days.

Table I. *Final weights and vitamin A liver reserves of 18 rats on three diets.*

Litter	Sex	Diet S (stock)		Diet C + vitamin A		Diet C	
		A	M	A	M	A	M
2561	♂	4,400	279	1100	202	0	177
	♀	19,000	190	1250	154	0	150
2574	♂	27,500	367	1750	260	0	225
	♀	33,000	334	1300	280	c. 100	270
	♀	26,000	248	1550	178	c. 200	170
2566	♀	14,000	230	c. 350	198	0	180
Averages	♂	16,200	327	1400	247	30	224
	♀	16,500	223	1050	177	70	133
Both sexes		16,400	275	1250	212	50	195

M = Final body-weight of animal.

A = Vitamin A content (in mg. of "standard" oil) of animal's liver.

when they were suckling, their mothers must have been thoroughly well supplied with vitamin A. The relatively low reserves of the newly weaned animals constitute, therefore, a further illustration of a phenomenon to which Dann [1932] and others have called attention and seem to afford further support for their suggestion that the passage of vitamin A from mother to suckling is limited by the extent to which it can pass from the maternal organism into her milk supply. Dann has found (private communication) that the liver reserves of the young animals are still lower if they have no access to supplementary sources of vitamin A (liver, green leaves, *etc.*) after suckling.

Table I shows the final body-weight and the vitamin A content of the liver of each of the 18 experimental animals, as well as the average figures for body-weight and vitamin A content.

DISCUSSION.

These results afford further confirmation of some deficiency in the vitamin A-free diet, other than the absence of vitamin A, to which attention has already been called [Bacharach, 1933]. The ingestion of 50 mg. (C.L.O.) of vitamin A per day for 150 days certainly enabled the experimental animals to remain in positive vitamin A balance, since their liver reserves were considerably higher at the end of the experimental period than at the beginning, yet their growth was only slightly better than that of animals receiving the same basal diet but no supplementary source of vitamin A, and very markedly inferior to that of animals receiving a full stock diet. However, the animals which had received the full stock diet accumulated a far larger reserve of vitamin A than those which had received the supplemented vitamin A-free diet. A number of possible explanations of these phenomena could obviously be brought forward, but there is no experimental evidence either in favour or against any one or other of them. The animals receiving the full stock diet S were obtaining their vitamin A partly from the dried milk, from which they were presumably getting about 1 g. of butter-fat per day, and from the supplementary supply of liver; they were also receiving carotene, from the supplementary supplies of green leaves and possibly partly from the butter-fat. It is not, therefore, possible to say how much vitamin A, actual and potential, they were receiving per day; their liver reserves were about ten times as high as those of the animals in group 2 but it seems highly improbable that they were obtaining over ten times as much vitamin A. For the animals in group 2 we know with some exactness how much vitamin A was being consumed; at a level of 50 mg. per day they must have received a total supply of 7500 mg. Of this only 1/6th was found in the livers at the end of the experiment; some 85 %, therefore, of the vitamin A fed to these animals was either lost from the body unchanged in the faeces, or destroyed during its passage from the alimentary canal to the place of storage in the liver. In spite of the fact, therefore, that the animals were receiving a diet containing 10 % of fat, which is believed to facilitate the absorption of fat-soluble vitamins, they did not appear to have stored much more than 15 % of the vitamin A ingested.

For the animals in group 3 the average vitamin A reserve was half that found for the control group of 12 young animals referred to above, but the difference between the 50 and 100 mg. respectively found in the livers of the two groups was barely outside the experimental error of the colorimetric estimations made by us.

It is clear that the animals in group 1 are adequately supplied with vitamin A and all other dietary requirements, and that the animals in group 3 in spite of

their relatively low vitamin A reserve at the beginning of the experiment, and in spite of the fact that this reserve showed little change at the end of the experimental period, were able to continue on a vitamin A-free diet for a considerable period. The animals in group 2 occupied an intermediate position. They were able to accumulate an appreciable amount of vitamin A in their livers and to make somewhat better growth than the animals in group 3, and they would doubtless have continued growing more or less indefinitely, in the manner previously found for animals similarly fed [Bacharach, 1933]. Investigations now in progress have for their object a further attempt to improve diet C so as to secure that animals receiving it and a supplement of vitamin A shall show normal growth and, indeed, reproduction also, if this can be achieved, and also to secure that animals receiving diet C from an early age shall cease to grow sooner, and at a lower weight, than do most of the animals at present receiving it. Evidence is already to hand that placing the young suckling animals, with their mothers, on the basal diet at 9 or 10 days of age brings about a very great improvement in this second respect, and therefore raises still further queries as to the soundness of the present assumptions about the passage of vitamin A from the maternal to the embryonic and suckling organism, and the effect of vitamin A reserves in the young animals on their growth potentialities. Either some hitherto unsuspected phenomenon must be responsible for the relation between "running out" time and pre-weaning diet, or a vitamin A reserve of 100 mg. (C.L.O.) in the weaning rat is a significant factor in protecting it against vitamin A deficiency.

SUMMARY.

1. Three comparable groups of six animals were fed upon a stock diet, a vitamin A-supplemented vitamin A-free basal diet, and the basal diet, respectively.
2. In conformity with earlier findings, the animals in the first group grew more rapidly and reached a greater final weight, at the end of 150 days, than those in the second group. The animals in the third group had, in the same time, reached an inconveniently high weight, and barely ceased to grow.
3. The liver reserves of vitamin A in each animal were estimated by Moore's method. The values were consistent with the growth performance in the three groups, and were compared with those of young control animals.
4. The bearing of these findings is discussed, with special reference to problems of technique in vitamin A testing and to the relation between liver reserves of vitamin A in adult does and in their young and the subsequent growth performance of the offspring, when these are given a vitamin A-free diet.

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Note added February 1st, 1933. Since this paper was written, we have heard from Dr V. Klemola that he has concluded the histological examination of all 18 rats, and that the results will in due course be published, along with subsequent findings carried out with further experimental material. Here it must suffice to quote his summary. "The histological changes between animals receiving diets S and C (unsupplemented) were marked. The latter showed a diffused distribution of lymphocytes in the follicular patches, whereas the former showed the normal compact structure, with clearly differentiated central "reaction centres." The hyperkeratinisation shown by the epithelial layers of skin, fore-stomach, *etc.*, was typical of the pathology of deficiency, as also was the diminution in hair growth, the reduction of length and thickness of fibre being signs of involution. The animals receiving the supplemented diet C showed normal characteristics, except in two cases where the lymphatic reticular structure was not quite normal in appearance, though that departure from normality may quite possibly be due to *post-mortem* changes." These findings support the view that the animals receiving unsupplemented diet C were indeed in a state of definite vitamin A hypovitaminosis, and that the failure of the animals receiving supplemented diet C to show as good growth performance as the animals receiving diet S was associated with some deficiency other than vitamin A shortage.