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

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SOME years ago a series of investigations was begun in these laboratories with the object of achieving as close an approximation as possible to the "ideal" basic diet for vitamin assay, and in particular for the assay of vitamin A by the "curative" procedure generally used. Two papers were published [Bacharach, 1933; Bacharach & Lester Smith, 1933] to describe our earlier results. In 1934 we began to introduce further modifications into our vitamin A-free basal diet; by 1936 we regularly employed a diet having the following composition:

Diet F		
	Parts	
Rice starch	50	} Fed as a paste with water. Every animal receives also 0.375 μ g. of calciferol per week
Arachis oil	10	
Casein (alcohol-extracted)	20	
Salt mixture*	5	
Dried yeast	5	
Yeast extract	2.5	
Wheat germ (fat-free)	2.5	

* No. 6, according to de Loureiro [1931].

This was found to give a more constant—and shorter—period of "running out", a higher incidence of xerophthalmia and, at the same time, a greater response to curative doses than the diets described in our two earlier papers, and has now been in continuous use since 1936.

The improvement in the diet is, in our opinion, due mainly to two causes. First, the constituents carrying vitamin B₁ and the vitamin B₂ complex have been increased from 5% in diets A and B [Bacharach, 1933] to 10% in diets C and F (2.5% yeast extract is included to increase general palatability and ease of mixing, although it is a poorer source of water-soluble vitamins than an equivalent weight of the solids of dried yeast). A modified diet F containing an extra 5% of dried yeast was found by experiment to be in no way superior to diet F, which we therefore consider to contain at least optimal amounts of the necessary water-soluble vitamins. Secondly, alcohol extraction removes traces of vitamin A from the casein without seriously affecting its biological value. Maitra & Moore [1939] have recently shown that "light-white" casein (that is, sodium caseinogenate) contains traces of vitamin A; it is almost certain that our casein contains similar traces before extraction with alcohol which is thus a more efficient method of removing such traces than is dry heating, besides being free from other objections.

We were interested to discover how far diet F approached the "ideal" laid down in our earlier paper. Would it, in fact, if supplemented with adequate vitamin A, permit young stock animals at least to grow normally, if not also to achieve normal fertility and a normal life-span?

Preliminary experiments suggested some residual inadequacy of diet F. The difference between animals receiving the diet with a (relatively) large supplement of vitamin A and those receiving our stock diet was shown in growth rates differing appreciably and probably significantly, though this difference was much less than was obtained in a comparison of the earlier diet C with the stock diet. Careful scrutiny of the constituents of diet F had failed to indicate any obvious dietary deficiency, when, for us, a most fortunate discussion of the whole question with Prof. A. Sordelli caused him to suggest an investigation into the relative palatabilities, as judged by the consumptions, of the two diets under apparently identical conditions. Although a little alarmed at the prospect, we decided to undertake the paired-feeding experiment which, it seemed to us, alone could settle the question.

Actually, "triad-feeding" was necessary, so as to have three strictly comparable groups of animals receiving, respectively, unlimited, unlimited diet F (with carotene supplement), stock diet limited to the intake level of diet F and unlimited stock diet. The two diets had almost exactly equal calorific values. The animals on diet F received a daily ration of 20 μ g. purified β -carotene. There were 12 males and 12 females in each group except the *ad libitum* stock diet group, for which only 11 females were available. In Table 1 the results have been given for the two sexes separately. In Fig. 1, combined growth curves are shown.

Table 1

	Vitamin A-free diet <i>ad lib.</i> + carotene		Stock diet limited		Stock diet <i>ad lib.</i>	
	♂	♀	♂	♀	♂	♀
Total food intake (g.) per animal in 100 days	1212	1065	1207	992	1384	1164
Average wt. increase (g.) in 100 days	210	129	216	133	238	135
s.d. of individual wt. increases	23.6	7.6	25.0	14.8	35.1	21.3

Fig. 1. Increase of body-weight in 100 days. S_U , mean values for 12♂ and 11♀ receiving stock diet F *ad lib.* S_L , mean values for 12♂ and 12♀ receiving stock diet, limited in amount to average intake of diet F. F , mean values for 12♂ and 12♀ receiving diet F *ad lib.*, with about 25 I.U. of β -carotene per animal per day.

The difference in mean weight-increases of male animals receiving unlimited or limited amounts of stock diet shows a degree of significance corresponding with a value of $P=0.09$, barely sufficient to justify connecting the distinct

difference in food intakes with the apparent difference in growth-rates. No other even nearly significant differences are to be found between the effects of pairs of diets.

We concluded, therefore, that we were justified in considering diet F a good approximation to the "ideal" basal diet, differing mainly, if at all, in a somewhat lowered palatability, and a consequent slightly reduced intake, from our stock diet which is known to have been adequate for health and fertility over many generations.

Use of diet F in many vitamin A assays has led to fairly satisfactory results: as has been pointed out by several workers, particularly by Richards & Simpson [1934], the nature of the "curative" vitamin A test makes the use of sick animals almost inevitable, and thereby introduces an inevitable source of error and uncertainty. Any proposals to improve still further basal diets are closely scrutinized by all workers in the field, and we learned with interest of the advantages claimed by Tainsh & Wilkinson¹ [1939] for the replacement of part of the basal diet by its equivalent (in protein nitrogen) of coconut meal. We decided to test this claim and at the same time to take the opportunity of making certain other observations, especially of comparing the relative values of male and female animals of our stock under our experimental conditions as "reagents" for vitamin A, and also to obtain an estimate of the probable limits of error obtainable in assays carried out by us. The composition of the modified diet (diet G) was as follows:

Diet G		
	Parts	
Coconut meal	30	} Calciferol supplement as with diet F
Rice starch	25	
Casein (alcohol-extracted)	15	
Arachis oil	10	
Salt mixture*	5	
Dried yeast	5	
Yeast extract	2.5	

* As in Diet F.

For reasons not germane to the nature of the experiment, the fat-free wheat germ (2.5%) was omitted from this diet. This slightly reduced the intake of aneurin and of certain constituents of the vitamin B₂ complex, but any difference in effect of the two diets can only have been in very small measure due to such a change; this is shown by the behaviour of the negative controls, for which diet F actually appeared more drastically deficient than diet G (see below). 10 g. of either diet furnished between 15 and 30 μ g. (5 to 10 I.U.) of aneurin per day; vitamin B₁ shortage is not, therefore, a limiting factor in either. The proximate analyses (calculated) of the two diets were:

	Diet F (%)	Diet G (%)
Fat	10.6	13.1
Protein	25.8	25.3
Carbohydrate	50.0	43.3
Ash	6.1	7.2
Fibre	—	3.3
Moisture	7.1	7.4
Calories per kg.	4085	4020

The number and distribution of the animals used in evaluating the results, as well as the assay figures obtained for the sample of oil and the probable limits of error of these figures, are given in Table 2. All the results were calculated by the

¹ We are much indebted to these authors for information about their results some months before publication.

Table 2

Diet	Sex	No. of animals	I.U. of vitamin A found per g. ($\bar{A}$)	σ_M^*	b^*	Probable limits of assay error†			
						$P=0.99$		$P=0.95$	
						%	I.U. per g.	%	I.U. per g.
Routine	♂	48	770	0.0719	63.1	65.5-153	505-1185	72.5-138	560-1070
Modified		56	830	0.0912	34.6	58.0-172	485-1430	66.5-151	550-1250
Routine	♀	52	507	0.1230	25.3	48 -207.5	245-1050	57.5-174	290-885
Modified		56	510	0.4497	7.5	7 -1440	35-7340	13 -760	67-3880

* $M = \log \bar{A} - 3$.

† Calculated from the data of the experiment itself.

Response = $a + b \log \text{dose}$.

method of Irwin [1937]. The doses of oil fed (twice a week) were equivalent to daily doses of 1.84 and 3.67 mg., the doses of Standard to 2 and 4 I.U. Every set¹ of isogenic animals was represented by one animal in each dosage group and in some instances by a negative control. The period of test was 5 weeks. Only those sets of isogenic animals in which all 4 dosed animals survived the test period without loss in weight have been included for purposes of calculation. Actually there were 5 males that died on the routine diet F, and 2 females that lost weight, but survived; the corresponding figures for the modified diet were 2 males, and 1 male and 1 female. One set of females on the modified diet has been omitted because of an accidental injury to one animal in the set.

Tests for significance have revealed certain points that bear on the questions raised earlier in this paper. The error of each of the 4 assays—taking the 2 diets and the 2 sexes separately—has been calculated from the responses obtained, according to the method of Irwin [1937]. No one of the assay values differs significantly from any of the others, though clearly those obtained with male rats are more trustworthy than those obtained with females. Further, the introduction of coconut meal into diet F and its transformation thereby into modified diet G has been accompanied by a marked lowering of its utility, especially when female rats are being used. It has, in fact, made females useless for the assay. Our finding as to the relative superiority of bucks over does for vitamin A assay—and the explanation of this superiority—is in good agreement, in spite of our different attack on the problem, with that of Coward [1932]. We have also compared our results with the findings of Coward [1933] as to the effect of the length of a test on its accuracy. We have calculated the values obtained with male rats on our routine diet for the 3-week, as well as for the 5-week, period and have found (Table 3) no increase in accuracy as the result of prolonging the test. In this respect, we differ slightly from Coward [1933] who obtained an increase in accuracy with the longer test period, but not sufficient to make worth while the extra work involved.

More detailed information about the responses given by the various groups of animals is summarized in Table 4, where the effects of modifying the diet and of the sex of the experimental animals are shown on the standard deviations of the responses and on the slopes of the various contributory response curves as well as on the standard deviations of these slopes. The lower errors, are, in part, associated with lower mean responses, but their correlation is found not to be significant; calculation of the coefficients of variation (the standard deviations expressed as percentages of the mean responses) shows no evidence of any trend.

¹ It is suggested that, to avoid a source of confusion from which the literature is by no means free, the word "group" be strictly confined in this kind of experiment to animals receiving the same dose, while the terms "litter", "set", "series" etc., as applicable, be used for the collection of individual animals over which the different doses are distributed.

Table 3

Length of test weeks	Value of b	Result of assay		Probable limits of error	
		I.U. per g. =antilog ($M+3$)	σ_M	$P=0.95$	$P=0.99$
3	42.6	814	0.0716	72.5-138	65.5-153
5	63.1	772	0.0719	72.5-138	65.5-153

Table 4

Diet	No. and sex of animals on each dose	Dose	Weight increases (5 weeks)				Slope of contributory response curves, b (with standard errors, σ_b)	
			Mean increase		Standard deviation of increase (σ_W)		Vitamin A	Carotene
			Vitamin A	Carotene	Vitamin A	Carotene		
Routine	12 ♂	Upper	58.5	68.3	14.8	16.8	61.7	64.5
		Lower	39.9	48.9	18.1	15.7	(22.7)	(21.9)
Modified	14 ♂	Upper	54.7	57.8	10.9	14.8	44.9	34.3
		Lower	41.2	47.4	11.6	18.7	(14.8)	(21.3)
Routine	13 ♀	Upper	41.7	46.6	5.25	7.4	38.8	13.7
		Lower	30.5	42.5	7.2	9.7	(8.3)	(11.4)
Modified	14 ♀	Upper	38.2	39.2	17.0	9.4	12.3	2.6
		Lower	34.5	38.4	8.8	11.8	(17.2)	(13.2)

The females on the routine diet showed a coefficient of 19%, the males on the modified diet 29% and both the other two groups of animals 32%; the coefficient was higher for the lower doses and slightly lower for the routine diet than for the modified diet.

Table 4 makes clear, however, that the main effect of sex and diet modification has been on the values of b , the slopes of the contributory and mean response curves. The "discrimination" shown by the animals—that is to say, the extent of the difference in response to a given difference in doses—was greater for males than for females, and greater on the routine diet than on the modified diet. In 3 out of 4 instances, males on the modified diet and females on either diet, the value of b was higher for vitamin A than for carotene. In this respect our findings appear to differ from those of Mead *et al.* [1939], who obtained no such difference under the conditions of their experiments, but to confirm the results of Tainsh & Wilkinson, quoted in those authors' paper. When, however, the standard errors of b are calculated for all the relevant pairs of comparisons, we find that the values of b only differ significantly for males and females. The difference for the values on routine and modified diets just fails to reach the 5% level of significance; the difference for test substance and carotene is not significant.

Females on the modified diet failed to discriminate significantly between the two doses (in the ratio of 2:1) either of vitamin A ($P=0.18$) or of carotene ($P=0.75$) and females on the routine diet showed discrimination of doubtful significance ($P=0.07$) between the two doses of carotene. Other authors have noted the reduction in the error of vitamin A assays to be obtained by using only males; thus Coward *et al.* [1931] point out that the value of b , the slope of the response curve, is greater for males than for females, so that the value of σ_M , the standard deviation of the logarithm of the result of an assay, must be greater with females than with males, provided, of course, that a lower standard deviation of response by females does not set off the difference in slope. Our figures confirm Coward's finding that the standard deviation of response (increase in body-weight) is less for females than for males; the values for the mean standard

deviation (weighted) are, for males 17.3 and for females 10.7. Thus our results indicate that, even with the basal diets giving the higher values of *b*, the difference in utility between males and females is mainly due to their difference in "discrimination", any difference in the values for the standard deviations of the responses being insufficient to compensate for the difference in slope of the response curves.

Examination of the protocols for negative control animals again points, albeit only slightly, to the superiority, that is, greater success in producing complete vitamin A deficiency, of the routine diet over the modified. (As negative control animals are included all animals that either received no supplement at all during the experimental period—"running out" time plus 35 days—or died within 7 days after receiving the first dose: nearly all the animals were in the former category. Such animals from the few litters not used in calculating the assay values have been included.) The protocols are summarized in Table 5. Animals of

Table 5. *Effect of diets on negative controls*

Sex	Diet	Mean no. of days* to death	Maximum gain* in weight	Mean gain* in weight at death	Incidence of xerophthalmia %
♂ ♀ ♂ ♀	Routine	38 (15)†	53 (13)	40 (13)	67 (12)‡
	Modified	48 (12)†	57 (12)	46 (12)	46 (13)
	Routine	43 (11)	51 (11)	37 (11)	73 (11)
	Modified	45 (18)	49 (18)	37 (18)	67 (18)

No. of animals in brackets.

* From weaning, at 20–25 days old.

† For the difference of these two means, $P=0.006$.

‡ 83%, including two animals with eye abscesses.

either sex die slightly earlier and show a slightly higher incidence of xerophthalmia on the routine diet than on the modified, though most of the differences appear to have a very low degree of statistical significance. The value of P , however, for the difference in mean ages at death of the two groups of male animals is 0.006, indicating a highly significant difference.

Some, if not all, of the results of this experiment that have been discussed might be attributed to the presence of traces of vitamin A in the coconut meal, but such an occurrence can hardly have any bearing on sex difference in response to doses of vitamin A. Speculation as to the relation of these differences in "slope" or discrimination, and still more of the differences in mean response, to the metabolism of carotene and vitamin A does not seem to us likely to serve a very useful purpose. We have been concerned rather with a study of methods of assay than of physiological processes. The results are presented in the hope that they may assist others who are involved in the difficult task of attempting to carry out quantitative measurement with biological material showing as low a variability as can be brought about by nature (inbreeding) and nurture (standardization of conditions before and during the experimental period).

It would seem pertinent in this connexion to refer here to certain other results obtained as a result of a large-scale experiment carried out by us some 3 years ago. These results serve to throw into relief a fact that is generally recognized, yet appears frequently to be overlooked by investigators making well-meaning efforts to increase the accuracy of biological tests to an extent rendered impossible by the nature of the experimental material.

In our earlier paper [Bacharach, 1933] we described broadly the genetic and environmental history of our experimental animals; we venture to claim that the

intensity of our drive towards uniformity could hardly be increased. Yet examination of the variability of our ordinary stock on a full diet, described in our earlier paper, shows how large a part of their variability under experimental conditions in assay work must be regarded as unavoidable. This is a matter that, like so many others of importance to bio-assayists, has already received the critical attention of Coward [1932]. She found the standard deviation of the weight-increase of vitamin A-deficient animals receiving test doses to be the same as that of the weight-increase of rats growing normally from the same average initial weight and for the same period. It seemed a matter of some interest, then, to examine the basic variability of normal animals.

105 young males were taken from their mothers at weaning, in groups of 3 per litter. They were maintained until they were 120 days old on the same stock diet as that received by their mothers. They were weighed twice weekly and their weights at 10-day intervals estimated by interpolation. On plotting the mean of these weights a smooth average growth curve was obtained; this may be taken as representative of our male stock and its environment at the time of the experiment, and is shown in Fig. 2. By the end of the experiment the following figures were obtained:

Fig. 2. Body-weight. Mean values for 105 ♂ receiving stock diet *ad lib.*

No. of animals (males)	105
Range of weights (g.)	205-378
Mean weight $\bar{W}$	297
$\sigma_{\bar{W}}$	30.3
$\epsilon_{\bar{W}}$	2.96
Coefficient of variation	10.2
$\bar{W} \pm 2/3 \times \sigma_{\bar{W}}$	277; 317
$\bar{W} \pm \sigma_{\bar{W}}$	267; 327
$\bar{W} \pm 2 \times \sigma_{\bar{W}}$	236; 358

Thus, over half the animals will differ by more than 20 g. from the mean; one third will differ by more than 30 g. from the mean. Of every 22 animals one will differ by 60 g. from the mean.

Examination of the individual protocols failed to reveal any appearance of correlation between size of litter from which the triad had been taken and initial or final weight of the animals, or between these weights and the age (in days) at weaning, that is, removal from the mother. (Young rats begin to consume food for themselves several days before the usual "weaning" time of 20-23 days.)

It did, however, appear that there was a marked correlation between the initial and final weights of an animal. The animals were accordingly divided into two series, which had differed slightly in pre-experimental history, because the differences between the mean weights at 30 days old were significant ($P=0.001$), although those at 120 days were not ($P=0.45$). It was not legitimate, therefore, to test the correlation between initial and final weights on all the animals treated as one series, and the correlation coefficients were accordingly calculated for the

two series separately. The figures for initial and final body-weights are shown in Table 6. It will be noted that the animals in Series I remain consistently ahead of those in Series II, although the difference has ceased to be significant at 120 days, because of the intervening great increase in weights. These figures—and others in our possession—point clearly to the lasting effect of initial differences in weight; a poorer beginning confers a permanent handicap.

Table 6

		Body-weight (g.)				
		30 days		120 days		
Series	No. of animals	Mean	σ_W	Mean	σ_W	<i>P</i> - for correlation of initial and final wt.
I	51	61.5	9.43	299.1	35.37	≤ 0.001 ($r = 0.7914$)
II	54	55.8	7.33	294.9	24.49	0.005 ($r = 0.4096$)
<i>P</i> - for difference between means		0.001		0.45		
		Body-weight (g.) at 30 days old		Increase in body-weight between 30 and 120 days		
Series	No. of animals	Mean	σ_W	Mean	σ_{dW}	<i>P</i> - for correlation of initial wt. and wt. increase
I	51	61.5	9.43	238.7	28.00	0.005 ($r = 0.4100$)
II	54	55.8	7.33	238.9	22.31	No correlation ($r = 0.1074$)
<i>P</i> - for difference between means		0.001		No significant difference		

In tests involving the use of weight increases, a more important consideration is the relation, if any, between the initial weight of an animal and its subsequent increase in weight. Inspection of the individual figures suggested that a correlation of this kind would be found in animals in Series I; calculation of the correlation coefficient showed this to be so. On the other hand, there was no correlation at all between initial weight and increase in weight of the animals in Series II. The animals in Series I were taken from our general breeding stock, and we have long regarded animals of this type as unsuitable for vitamin A assay, owing to their reserves of vitamin A being too varied and rather too high. The animals in Series II had, along with their mothers, undergone a pre-experimental period of vitamin A deficiency from about the 10th day after birth; further, the mothers themselves had, before maturity, been submitted to a vitamin A-deficient diet for 2-3 weeks. Only animals raised in this manner are used by us for work on vitamin A: the procedure is partly based on a recommendation of Nelson [1928], to which reference was made in the earlier papers of this series.

It will be observed also that the standard deviation of the values for the males in Series II is less than that of the values in Series I; the difference has a significance level of only 20% and can therefore be disregarded. Analysis of the results obtained with two similar groups of females (51 in Series I and 48 in Series II) also showed a difference in standard deviation. Here the animals in Series I varied less than those in Series II and the difference has a 1% level of significance. It is difficult to understand how a procedure—pre-experimental restriction of ingested vitamin A—which has had no effect on the variability of the males can have increased the variability of females.

On the other hand, the correlations observed with males were exactly paralleled in the females. In Series I initial weight was strongly correlated with increase in weight ($r=0.343$; $P=0.015$). In Series II this correlation disappeared; indeed it became slightly, though not significantly, negative ($r=-0.073$). There seems little doubt, therefore, that the pre-experimental treatment of both males and females has had the same effect: cutting down vitamin A reserves to rock-bottom has eliminated—at any rate for animals receiving a full stock diet—the normally marked effect of original weight on rate of growth (weight-increase) during the 5th to 16th weeks of life. There seems no reason to doubt that the effect in question would also be eliminated in animals receiving a vitamin A-free diet.

The practical conclusion would seem to be that, if animals are taken direct from unprepared stock for tests involving increases in weight, then they should be arranged in sets of identical initial weight. This conclusion is, in itself, a justification for using litter-mate (isogenic) sets, because variations in weight of animals of the same sex and litter can be seen from a large number of records, our own and others, to be much less than the variations between the mean weights of groups of isogenic animals. In vitamin A assays, however, this special arrangement of the animals may be unnecessary, provided that a suitable depletion period has secured that the weight increases of the animals are independent of their initial weights.

Although the figures given in the later part of this paper are derived from a relatively large sample of normal animals, they indicate to how great an extent the variance of a smaller group of animals, receiving an experimental—that is, a deficient—diet, is due to inherent variability that is entirely independent of the experimental conditions imposed during a vitamin assay.

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