73. VITAMIN A STUDIES WITH RATS AND PIGS

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MANY aspects of the metabolism of vitamin A and of its precursors are still insufficiently known; this is especially true with regard to information available for animals other than the rat. Two of us (A. S. F. and S. K. K.) have for some time been interested in the importance of vitamin A in pig feeding. For reasons both practical and scientific we wanted to gain more knowledge of the utilization by the pig of certain carotenoids, of the relative value for the pig of carotene and of preformed vitamin A and of the minimum vitamin A requirements of this animal. Another of the authors (T. H. M.) has studied on rats the biological activity of vitamin A alcohol and of natural vitamin A esters and further comparisons using a different animal were thought likely to be of value. The present joint paper gives the results of all these inquiries. The biological tests were carried out at Shinfield and the chemical and physical manipulations were mostly done in the laboratories of The British Drug Houses, Ltd.

It is generally accepted that in order to exhibit vitamin A activity a carotenoid pigment must contain a β -ionone ring or rings. Recently, however, Lund [1938] and Petersen [1939] reported that zeaxanthin was effective in preventing symptoms of vitamin A deficiency in pigs, but that 'xanthophyll' (lutein) was inactive in this respect. If confirmed, this finding would be of considerable importance, not only because it would demonstrate the unique ability of the pig to convert a dihydroxycarotene into vitamin A, but also from the practical standpoint of pig feeding. Maize, an important pig food, contains relatively more zeaxanthin than carotene and cryptoxanthin [Kuhn & Grundman, 1934]. The observation of Lund [1938] that the pig can make use of dihydroxy- β -carotene but not of dihydroxy- α -carotene (lutein, xanthophyll) is certainly puzzling, as it implies that a hydroxy- β -ionone ring is utilized in one case but not in the other. The preparation of zeaxanthin used by Lund [1938] was admittedly contaminated with carotene, and we felt that for this and other reasons a re-investigation of the value of zeaxanthin as a source of vitamin A for pigs was amply justified.

Pure β -carotene has, by definition, a potency of 1.67×10^6 I.U./g., while, according to most recent data [Holmes & Corbett, 1937; Mead *et al.* 1939; Baxter & Robeson, 1940], the potency of pure vitamin A measured on the rat is nearly double this figure, i.e. about 3.0 – 3.3×10^6 I.U./g. These figures indicate that this animal, even under most favourable conditions, can form only one molecule of vitamin A from one molecule of β -carotene. This is what one would expect on theoretical grounds, as there is no reason to suppose that the fission of the β -carotene molecule takes place symmetrically [Morton, 1940].

The information regarding other animals is less definite, but Guilbert *et al.* [1940] have shown that cattle, sheep, pigs and horses are, if anything, less efficient than rats in converting carotene into vitamin A. Full references to

this work will be found in a recent paper by Hart [1940]. It was found that to cover minimum requirements the four species needed 5.3–7.1 $\mu\text{g.}$ of vitamin A¹ per kg. body weight but that 24–34 $\mu\text{g.}$ of carotene, either from artificially dried lucerne or as a preparation (88% β - and 12% α -carotene) in oil, were required for the same purpose. The animals obtained therefore a yield of only about half a molecule of vitamin A from one molecule of β -carotene. At least 5–10 times the minimum level is recommended in practice by the American authors. In this country, Dunlop [1934] found that 14 mg. of carotene per 100 lb. of food are insufficient for normal growth of pigs, while 62 mg./100 lb. give normal growth with storage of the vitamin. These values correspond for a 50 kg. pig to 12 and 53 $\mu\text{g.}$ respectively of carotene per kg. body weight [Dunlop, 1933] and agree with the American experience. According to Lund [1938] the minimum requirement of pigs up to 40 kg. in weight does not exceed 30 $\mu\text{g./kg.}$ body weight.

The relation between the biological activity of materials containing vitamin A and their spectral absorption at 328 $m\mu$ remains uncertain in spite of the numerous investigations that have been devoted to this subject [e.g. Coward *et al.* 1931; 1932; Morgan *et al.* 1935; Hume, 1937; 1939; Moll & Reid, 1939]. Values ranging from below 1000 to over 3000 have been found for the factor for converting $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ to biological units, and statistical analyses have in some cases [Hume, 1939; Morgan *et al.* 1935] indicated that the differences between the factors assigned to different materials are greater than the variations to be expected on account of the errors inherent in biological tests.

Although a survey of the available results reveals no hard and fast relation between the type of material tested and its appropriate conversion factor, certain generalizations hold with few exceptions. Some commercial vitamin A concentrates prepared from fish liver oils by saponification tend to give low factors, as also do mammalian liver oils and the corresponding concentrates. Higher values are usually given by unhydrolysed fish liver oils and in particular by fresh medicinal cod-liver oils and by the reference oil of the United States Pharmacopoeia. Exceptionally high factors have been claimed by Moll & Reid [1939] for samples of a commercial vitamin A concentrate which is prepared without saponification.

A number of explanations for the wide variation in experimentally determined conversion factors have been suggested. Pritchard *et al.* [1937] have, for example, shown that mammalian liver oils contain a factor which contributes to spectral absorption at 328 $m\mu$ but has little biological activity. The presence of this material probably accounts for the low conversion factors found for mammalian liver oils. The low biological activity of some fish liver oils relative to their extinction coefficient at 328 $m\mu$ may similarly be due to the presence of oxidized vitamin A [Robinson, 1938], cyclized vitamin A [Edisbury *et al.* 1932; Embree, 1939], vitamin A₂, or possibly other substances having little or no biological activity. Another explanation is that different oils may contain different proportions of the many possible isomers of vitamin A.

Concentrates prepared by saponification have most frequently been found to give the lowest conversion factors and it is therefore conceivable that this operation affects the potency of the vitamin. Since the vitamin A in fish liver oils is esterified, while that in concentrates prepared by saponification is free

¹ Wherever quantities of vitamin A are in this paper expressed in terms of weight it has been assumed that pure vitamin A has $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ = 1800 [Mead *et al.* 1939; Baxter & Robeson, 1940]. Quantities quoted from the literature have been recalculated to this basis to facilitate comparison.

[Bacharach & Smith, 1928; Reti, 1935; Hamano, 1935; Hickman, 1937; Tischer, 1938], the question arises as to whether a lower conversion factor should be ascribed to vitamin A alcohol than to vitamin A esters.

The existing evidence consists for the most part in experiments in which crude fish oils and crude concentrates were assayed against β -carotene on rats. In 1936 careful assays of a sample of halibut liver oil and of its non-saponifiable fraction were carried out in ten laboratories. Although the results [Hume, 1937; 1939] showed a higher conversion factor for the oil than for the concentrate, the latter was found by the spectrographic test to have deteriorated during the test period by an amount sufficient to account for the discrepancy. Emmet & Bird [1937] reported that tests carried out on a fish liver oil and on concentrates prepared from it by varying degrees of saponification indicated that a lowering of conversion factor occurred which ran parallel with the progress of hydrolysis. Hickman [1939] stated that distilled esters of vitamin A had the same conversion factor as the vitamin A in crude oils, whereas concentrates prepared by saponification always had a smaller factor which might be less than half that of the oils. Moll & Reid [1939], using a bioassay technique differing greatly from that normally used in England, found conversion factors of from 3500 to 3700 for a commercial vitamin A concentrate containing vitamin A esters. To the non-saponifiable fraction of this concentrate they assigned a factor of 1800.

The problem under discussion is complicated by the following considerations: (a) vitamin A alcohol in concentrates is more readily oxidized than vitamin A esters present in fish liver oils; (b) oxidation products of vitamin A absorb strongly in the neighbourhood of $328\text{ m}\mu$ but have little or no biological activity [Edisbury *et al.* 1932; Robinson, 1938]. It is therefore to be expected that low conversion factors will be found for commercial vitamin A concentrates which may have become partially oxidized during preparation and storage, as well as for concentrates which have been carefully prepared in the laboratory but which have not been fully protected against oxidation during the bioassay period. It is evident that the conversion factor applicable to any material containing vitamin A depends in the first place on its content of impurities affecting the spectrographic test. It follows that evidence of the true relation between the biological activities of free and esterified vitamin A is obtainable only from tests on materials free from such impurities.

One object of the present experiment was to discover whether any difference could be detected between the potencies of vitamin A alcohol and natural vitamin A esters when both had been freed as completely as possible from spectrographic impurities by molecular distillation. Simultaneous tests of the purified materials were carried out on pigs and on rats. A more comprehensive comparison of similarly purified materials was carried out on rats by Dr Underhill at The British Drug Houses, Ltd., before the experiment on pigs was undertaken. His results will form the subject of a separate paper.

EXPERIMENTAL

Preparation of materials

Vitamin A alcohol and purified natural vitamin A esters. Our principal object in the purification of these materials was to eliminate as completely as possible substances such as oxidized and cyclized vitamin A which give rise to irrelevant absorption at $328\text{ m}\mu$.

Vitamin A alcohol having $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ = 1600 was used for these experiments. It was prepared as described by Mead *et al.* [1939] by molecular distillation of the non-saponifiable fraction from a rich fish liver oil.

The natural vitamin A esters were made by purifying an ester concentrate obtained by distilling unhydrolysed fish liver oil. The crude material, a dark brown semi-solid (Carr-Price value 21,000), was dissolved in light petroleum and the solution was filtered through a column of activated alumina. The column was rinsed with the solvent until the brown zone, which at first occupied the top half, reached nearly to the bottom. The filtrate and rinsings were concentrated under diminished pressure, leaving a pale yellow oil which was distilled in a cyclic molecular still of the Hickman type. A first fraction consisting of a small quantity of red oil containing some cyclized vitamin A and vitamin A alcohol was obtained by circulating the distilland over the distilling surface successively at 80°, 90°, 100°, 120°, 130°, 140°, 150° and 155°. A second fraction consisting of vitamin A esters contaminated with sterols and traces of vitamin A alcohol was removed by cycles at 160°, 165° and 170°. The third fraction, which was used to prepare the feeding solutions for this experiment, was collected by circulation once at 175° and twice at 180°. It consisted of a pale yellow oil having Carr-Price value 33,000 and $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ = 660.

If pure vitamin A alcohol has an extinction coefficient $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ = 1800, this concentrate of the natural esters contained 36.7 % of vitamin A. On the assumption that the average molecular weight of the fatty acids combined with the vitamin A is of the same order as that of oleic acid, the vitamin A ester concentrate contained about 73 % of natural vitamin A esters. The remainder probably consisted chiefly of fatty triglycerides and fatty acid esters of cholesterol.

β -Carotene. A specimen of commercial crystalline mixed carotenes was purified by chromatographic adsorption on magnesia followed by crystallization from benzene-ethanol. At no time during the preparation was the substance exposed to a temperature exceeding 45°. The product had M.P. 184° (corr.) and $E_{1\text{cm.}}^{1\%}$ 456 $m\mu$ = 2400 (in cyclohexane).

Zeaxanthin was obtained by hydrolysis of the naturally occurring dipalmitate (physalien). The hydrolysate was carefully separated by chromatography from carotene, cryptoxanthin and other carotenoids present in crude extracts of *Physalis*. The seed pods from freshly picked *Physalis* (approx. 30 kg.) were separated from the berries, covered immediately with alcohol and set aside for a month. The alcohol was run off and the pods were air-dried, first at room temperature and finally overnight at 37°, before being pulverized in a ball-mill from which the air had been displaced by CO₂. The powder was immediately extracted with successive portions of light petroleum until all the pigment had been removed. The combined extracts were evaporated in an atmosphere of N₂ under diminished pressure to a small bulk; sufficient benzene was added to give a clear solution (vol. approximately 1.5 l.), which was treated with alcohol (4.5 l.). After a short time, the crude physalien was collected, washed with alcohol, and immediately recrystallized by the addition of warm alcohol to its solution in warm benzene. Yield 57 g. The physalien was hydrolysed and the crude zeaxanthin purified in several small batches as follows.

Physalien (12.5 g.) was dissolved in benzene (125 ml.). The solution was diluted with light petroleum (1875 ml.) and shaken for 8 hr. at room temperature with a solution of KOH (150 g.) in methyl alcohol (625 ml.). On the addition of distilled water (about 1 l.) the zeaxanthin separated at the interface between the petroleum and aqueous-methanol phases. The upper and lower layers were

siphoned off leaving the zeaxanthin suspended in a small volume of liquid from which it was separated by filtration. The product was thoroughly washed on the filter with 50 % methyl alcohol and dried in a desiccator. Yield 6.5 g. from 12.5 g. physalinen. For purification the crude material was dissolved in warm ethylene dichloride (3.5 l.) and the solution, after being cooled to room temperature, was filtered through a column (ca. 11 × 55 cm.) containing 3 kg. of 'heavy' magnesia. The chromatogram was developed with ethylene dichloride (2.5 l.) followed by ethylene dichloride mixed with 15 % benzene (1 l.) until the intermediate orange band containing the zeaxanthin was well separated from the zones above and below it. The appropriate zone was dissected out and eluted with chloroform containing methyl alcohol (5 %). The eluate was washed several times with water in order to remove methyl alcohol, dried (Na_2SO_4) and evaporated under diminished pressure to a small volume from which the zeaxanthin crystallized on the addition of light petroleum. After recrystallization from chloroform (150 ml.) the product melted at 214° (uncorr.). Yield 3.35 g. A solution in ethanol showed absorption bands at 454 and 480 $\text{m}\mu$:

$$E_{1\text{cm.}}^{1\%} 454 \text{ m}\mu = 2520. \quad E_{1\text{cm.}}^{1\%} 480 \text{ m}\mu = 2230.$$

Strain [1938] gives the following data for pure zeaxanthin (m.p. 212–213°). Absorption bands (in ethanol) at 423.5 (weak), 451.5 and 483 $\text{m}\mu$. The following extinction coefficients are taken from the curve on p. 102 of Strain's monograph:

$$E_{1\text{cm.}}^{1\%} 454 \text{ m}\mu = 2470. \quad E_{1\text{cm.}}^{1\%} 480 \text{ m}\mu = 2190.$$

Preparation and storage of the animal feeding solutions

Solvent oil. Arachis oil specially selected on account of low peroxide content and the negligible absorption given at 328 $\text{m}\mu$ was used for preparing the test solutions after treatment with quinol (0.01 %).

Solutions of vitamin A alcohol. In calculating the quantities to be used for making the solutions of vitamin A alcohol and vitamin A esters, it was assumed that the potency in I.U./g. is equal to the extinction coefficient multiplied by 1600. A solution containing approximately 500 I.U./g. for feeding to the pigs was prepared by dissolving 1.82 g. of vitamin A alcohol ($E_{1\text{cm.}}^{1\%} 328 \text{ m}\mu = 1600$) in 541.1 g. of arachis oil and diluting part of this solution (273.7 g.) to 4.87 kg. A solution containing 200 I.U./g. for the rat experiment was prepared from this by appropriate dilution with arachis oil. The extinction coefficients of the two solutions were measured immediately after preparation and again at intervals during the biological experiments. The results of these tests and of those on the solutions of vitamin A esters, β -carotene and zeaxanthin are given in Table 1.

Solutions of vitamin A esters. A solution containing 500 I.U./g. of the purified vitamin A esters in arachis oil was prepared by dilution in two stages in a manner similar to that used for making the vitamin A alcohol solution. From this the 200 I.U./g. solution for the rat experiment was prepared by dilution with the same solvent.

Solutions of β -carotene. The biological activity of the carotene was assumed to be 1,670,000 I.U./g. The following procedure was adopted in order to avoid the risk of decomposing the carotene by exposure to temperatures above 40°. β -Carotene (1.314 g.) was dissolved in pure benzene (300 ml.) and approximately one-third of this solution was added to about 1 l. of arachis oil contained in a 5 l. flask. The greater part of the benzene was removed in a vacuum and the solution transferred to a tared 10 l. flask. These operations were repeated twice, using the remaining two-thirds of the solution of carotene in benzene, the last

Table 1. *Spectrophotometric measurements on the feeding solutions*

Substance	Correct potency I.U./g.	Date	$E_{1\%}^{1\text{cm.}}$	I.U./g.
Vitamin A alcohol	500	7. ii. 40	0.303	485
			0.304	
		13. iii. 40	0.276	454
			0.292	
		1. v. 40	0.286	458
	200	1. viii. 40	0.295	465
			0.286	
		8. ii. 40	0.121	194
		12. iii. 40	0.115	185
Vitamin A esters	500	6. i. 40	0.300	488
			0.310	
		13. iii. 40	0.300	480
		1. v. 40	0.297	475
		1. viii. 40	0.271	435
	200		0.271	
		3. viii. 40*	0.295	473
		6. i. 40	0.121	194
		12. iii. 40	0.118	189
β -Carotene	500	3. i. 40	0.710	494
		13. iii. 40	0.750	522
		1. v. 40	0.750	522
		1. viii. 40	0.647	450
	200	6. i. 40	0.290	202
		12. iii. 40	0.285	198
Zeaxanthin	200†			
	1st batch	3. ii. 40	0.320	200
	2nd batch	27. iii. 40	0.305	191
	3rd batch	7. v. 40	0.301	188
	4th batch	6. vi. 40	0.286	179
	5th batch	1. vii. 40	0.303	189
	6th batch	22. vii. 40	0.285	179

* Fresh unopened bottle.

† Presumptive 'units' based on assumption that 1 mol. zeaxanthin has the activity of 1 mol. of β -carotene.

traces of which were rinsed in with a small quantity of the solvent. The 5 l. flask was finally washed several times with arachis oil and the washings were added to the contents of the 10 l. flask so as to bring the total weight of the solution to 4.38 kg. The last traces of benzene were removed by connecting the flask to a vacuum pump of large capacity while a slow stream of CO_2 from a submerged sintered-glass gas distributor passed through the solution. The temperature of the oil was raised to 40° during the later stages of this operation and care was taken to exclude light. A solution containing 200 I.U./g. for feeding to the rats was prepared from the stronger solution by dilution with arachis oil.

Solution of zeaxanthin. It was intended that some of the pigs should be given quite large doses of zeaxanthin in order that biological activity of a low order could be detected. Attempts were therefore made to prepare rather concentrated solutions in the hope that feeding of excess arachis oil to these pigs could be avoided. Owing, however, to the low solubility of zeaxanthin in oil, we were compelled to use a solution no stronger than 0.0128%. Such a solution would contain 200 I.U./g. if zeaxanthin had the same activity, molecule for molecule, as β -carotene. We discovered later that zeaxanthin crystallized slowly on storage at room temperature even from solutions as dilute as this. The resulting fall in concentration of the six batches of solution made during the course of the

experiment was estimated colorimetrically by means of a Leitz photometer at intervals during the period in which each batch was fed to the pigs. The loss, which never exceeded 10%, was considered to be negligible. Each batch weighed about 8 kg. and was prepared by a method similar to that used for preparing the carotene solution, except that chloroform was used in place of benzene because the latter is a very poor solvent for zeaxanthin. A portion of the first batch was used for the rat test. The batches were prepared as required in order to avoid long periods of storage.

Storage and stability of the feeding solutions. The solutions were kept under N₂ in a refrigerator. In spite of these precautions some deterioration occurred, as is shown in Table 1.

Levels of feeding

All substances tested were given to pigs and rats at two levels of intake. For pigs the low level was based on the estimates of minimum requirements put forward by Hart [1940], but to find out any differences all substances were fed at the same presumptive level of biological activity.

As the lower dose, 100 I.U. of vitamin A were given daily for 10 lb. live weight. This was equivalent to 7.7 μ g. of vitamin A (as alcohol or natural esters) and 13.3 μ g. of β -carotene per kg. body weight (*vide supra*). The higher dose for these substances was 300 I.U./10 lb. live weight. Zeaxanthin was presumed to be as active as β -carotene but was fed at levels of 300 and 1500 calculated I.U./10 lb. live weight. Details of rat tests are given separately on p. 703.

Tests on pigs

Methods. A 'normal' pig-meal mixture, similar to that used by us previously [Foot *et al.* 1938; 1939], was prepared for this experiment. It consisted of:

Barley meal ...	...	...	40
Weatings ...	...	...	45
Extracted groundnut cake...			5
Meat meal ...	...	...	6
Brewer's yeast ...	...	...	2
Ground limestone ...	...	...	1½
Common salt ...	...	...	½

Examination showed that the diet contained only traces of yellow pigment and this was mostly xanthophyll. No β -carotene was detected; the carotene content was certainly much less than 0.02 mg. per 100 g.

The general methods of management of the pigs were as already described [Foot *et al.* 1938; 1939].

Three litters of pigs were reared on this ration. The dams of each litter were given the meal shortly after farrowing and received this food alone during suckling. The young pigs were offered 4 parts of the meal mixture together with 1 part of dried skim milk powder from the time they were 3 weeks old; they were weaned when 8 weeks old and the three litters were then pooled, groups from them being selected for five treatments as follows:

- Group 1. 2 pigs as controls; no addition to meal mixture.
- Group 2. 6 pigs receiving zeaxanthin, 3 the higher and 3 the lower level.
- Group 3. 4 pigs receiving β -carotene, 2 the higher and 2 the lower level.
- Group 4. 4 pigs receiving vitamin A alcohol, 2 the higher and 2 the lower level.
- Group 5. 4 pigs receiving vitamin A ester, 2 the higher and 2 the lower level.

The grouping was carried out in such a way that the litters were as far as possible evenly distributed among treatments. In Fig. 1 and Tables 2 and 3 littermates are indicated by letters.

After grouping, the addition of skim-milk powder was discontinued and the meal alone was fed. Each pig was given in addition once a fortnight a solution of calciferol in oil supplying 500 I.U. of vitamin D daily. The pigs were group-fed twice daily. The amount of food allowed each pen of pigs was based on the live weight of each animal and was adjusted once a week after the animals had been weighed. The standard used was that usually recommended by the Pig Experiments Coordination Committee [Shorrocks, 1940]. When appetite was depressed so that the animals in any pen failed to eat the amount demanded by their live weight, they were fed according to appetite.

Once weekly, the pigs were given individually solutions containing their respective addenda. For this purpose, the pigs were placed one at a time in small wooden cages and offered the material mixed with a small quantity of meal. Owing to the large amount of zeaxanthin solution required by the three pigs receiving the higher level they were not separated but were given their zeaxanthin together during one feed each day.

Straw was used as litter in the first part of the experiment and wood shavings during the last few weeks.

Results

Preparation period. Some difficulty was experienced in the rearing and preparation of the experimental pigs. Two litters were reared with no more than normal losses during suckling. In one litter, however, six of the eight pigs alive after the first 27 days died during the suckling period. In most of these post-mortem examination showed inflammation of the intestines and pneumonia. The real cause of the losses was, nevertheless, obscure and there is little reason for attributing them solely to the diet at this early stage.

The vitamin A content of the livers of the animals which died during the preparation period is given in Table 2 (cf. p. 702). The data are few, but they

Table 2. *Vitamin A in livers of pigs lost during the suckling and weaning stage*

	Litter A (Sow 185)	Litter B (Sow 576)				Litter C (Sow 607)			
		17				11			
		11				8			
		Vitamin A in liver				Vitamin A in liver			
No. born	12								
No. alive at beginning of experiment	10								
		Age at death days	Wt. liver g.	µg. in whole liver µg./g.		Age at death days	Wt. liver g.	µg. in whole liver µg./g.	
Losses during prepara- tion period	None	39	154	9.6	1478	18	123	10.8	1328
		74	74	7.8	577	27	223	9.1	2029
		80	151	7.8	1178	32	151	11.3	1706
						49	297	7.0	2079
						54	166	2.8	465
						57	247	4.3	1062
Remaining for grouping	10	8				2			

show that the liver reserves at birth were small and they suggest that the amount was decreasing during suckling. The feeding of carotenoids or vitamin A was started when the pigs, according to litter, were 10 or 12 weeks old, and it is

reasonable to assume that at this point the experimental animals had little or no vitamin A in their livers.

Experimental period

Growth. The growth curves of the twenty experimental pigs for the period of dosing are shown in Fig. 1.

All the pigs receiving vitamin A alcohol and vitamin A ester grew consistently throughout the experimental period. They grew at about the normal rate for pigs receiving good fattening rations and there was no evidence of any advantage of the higher level of vitamin A over the lower.

Fig. 1. Growth curves of experimental pigs.

The two animals receiving the higher level of β -carotene also grew consistently and at a good rate. The growth of one on the lower level was, however, definitely subnormal and the animal eventually died 20 weeks after the commencement of the dosing.

The two control pigs were originally the biggest animals from two litters. They continued to grow, although in one case irregularly, for about 20 weeks. Growth then ceased and both pigs died a few days later.

There was much variation in the growth of the pigs receiving zeaxanthin. Two animals, one on the high level and one on the low level, grew normally for about 20 weeks. The first then became steady in weight and did not resume growth until given vitamin A, the second continued to grow at a very reduced rate. The remaining four pigs grew very little throughout the experiment; three of them died, two on the lower and one on the higher level; the fourth recovered after vitamin A treatment.

Health and symptoms. Of the twelve pigs which received vitamin A alcohol, vitamin A ester or β -carotene only one showed any symptoms or evidence of ill-health. This animal (no. 1383) was receiving the low level of β -carotene. Its appetite declined 15 weeks after the start of the experiment. It became thin, failed to thrive, developed laboured breathing and died 5 weeks later without showing any other symptoms.

The two negative control pigs showed no symptoms apart from occasional loss of appetite until the 20th week, when pig no. 1387 experienced a short convulsive fit from which it quickly recovered. The animal, however, became unstable on its legs and died 11 days later following two violent convulsive fits. The appetite of the other control pig, no. 1379, failed completely after the 21st week and the animal was unstable on its feet. Apart from that, however, no symptoms were shown and the pig died suddenly in the 22nd week.

The six zeaxanthin pigs all showed symptoms varying from only slight lack of appetite to typical convulsive fits. One animal, no. 1392, apparently became blind, while another, no. 1388, showed the typical irregular gait which we have often noted in previous experiments [Foot *et al.* 1938; 1939]. Hence the clinical evidence strongly suggested that the zeaxanthin at both the low and the high levels was biologically inactive.

Post-mortem findings. In the six pigs which died during the experiment, pneumonic changes, varying in intensity from slight congestion to grey hepatization, were the most conspicuous findings at necropsy. Patchy inflammation of the intestines, especially of the caecal region, was no less characteristic. This picture agrees with our earlier observations [Foot *et al.* 1938; 1939].

Curative treatment. Three pigs which failed to thrive when their diet was supplemented with zeaxanthin were given curative doses of vitamin A, 1500 I.U./10 lb. body weight daily. A spectacular cure was achieved in two cases (nos. 1384 and 1388); the third pig, which was obviously in a very poor state of health, failed to recover and died a few days after the curative attempt.

Vitamin A content of livers. The livers of all experimental pigs were examined for vitamin A by the method fully described by Foot *et al.* [1938]. The results are given in Table 3 expressed in micrograms of vitamin A. Values in brackets refer to animals which were given vitamin A as a curative measure. As a result of several estimations which agreed well together we have found that 1 Moore blue unit [Moore, 1930], as measured by us on vitamin A concentrates or on the dilutions used for pig feeding, is equivalent to 0.137 μ g. vitamin A. The values are quoted on the assumption that the same factor would apply to the vitamin A present in pig livers. Dr A. E. Gillam kindly undertook to assay the livers by the spectrographic method. Unfortunately the results were with two exceptions rendered inconclusive because the vitamin peak was obscured by irrelevant absorption. Good vitamin A curves, however, were obtained for the livers of pigs nos. 1377 and 1388, especially for the former. From the extinction coefficients at 328 $m\mu$ it was calculated that these livers contained 22.3 and 139 mg. of vitamin A respectively. The corresponding figures calculated from the blue value measurements were 25.6 and 72 mg.

The complete absence of vitamin A from the livers of the three pigs which received only zeaxanthin is of particular interest and confirms the clinical evidence that this carotenoid is biologically inactive. The livers of the two pigs receiving the lower dose of β -carotene contained little or no vitamin A while the reserves of those given 300 I.U. of β -carotene per 10 lb. live weight were rather less than those of pigs given only 100 I.U. as vitamin A alcohol or ester.

Table 3. *Vitamin A in livers of experimental pigs*

Pig no. and litter	Age at death days	Wt. of liver g.	Vitamin A in liver	
			$\mu\text{g./g.}$	$\mu\text{g. in whole liver}$
Control 1379 A	249	1889	0	0
1387 B	229	2604	0	0
Zeaxanthin (low level)				
1380 A	233	923	0	0
1385 A	248	1505	0	0
1388 B	290	2009	(36.0)*	(72,324)*
Zeaxanthin (high level)				
1384 A	359	1501	(4.5)*	(6,754)*
1386 B	290	1500	0	0
1392 B	269	1057	(2.3)*	(2,431)*
Vitamin A ester (low level)				
1382 A	238	2094	1.3	2,722
1396 C	247	1698	1.3	2,207
Vitamin A ester (high level)				
1377 A	238	1684	15.2	25,597
1390 B	248	1698	8.8	14,942
β -Carotene (low level)				
1383 A	233	1796	0	0
1393 B	248	1698	Faint trace	Trace
β -Carotene (high level)				
1378 A	238	1953	0.5	976
1389 B	227	1698	0.3	509
Vitamin A alcohol (low level)				
1381 A	238	2066	1.4	2,892
1397 C	247	1868	0.9	1,681
Vitamin A alcohol (high level)				
1376 A	238	1670	9.1	15,197
1391 B	248	1613	7.8	12,581

* After receiving vitamin A.

Tests on rats

The substances given to pigs were also assayed on rats in curative tests. The technique was as described by Booth *et al.* [1934], with the minor modifications that dextrinized potato starch was used instead of dextrinized rice starch, that de Loureiro's [1931] salt mixture was used and that a small fraction of the yeast present in the diet was irradiated to supply vitamin D. The tests were of 4 weeks' duration. All test substances were given twice weekly, β -carotene, vitamin A alcohol and vitamin A ester at two levels of 2 and 4 I.U. daily and zeaxanthin at the presumptive levels of 4 and 12 I.U. (cf. p. 699). Groups of four male and five female rats were used for each substance. The results are given in Table 4.

Only three out of nine animals receiving the lower level of zeaxanthin survived the experimental period. There were two deaths at the higher level and the remaining seven rats lost weight heavily. The results show that zeaxanthin is of no value for the rat as provitamin A.

All rats receiving vitamin A alcohol, vitamin A ester and β -carotene survived, and gains in weight at corresponding levels of intake were very similar, indicating that all three substances had the same biological activity.

Table 4. *Weight gains of vitamin A-deficient rats receiving various supplements*

4 I.U. Zeaxanthin				12 I.U.			
Rat no.	Initial wt. (g.)	4 weeks		Rat no.	Initial wt. (g.)	4 weeks	
		Wt. (g.)	Gain (g.)			Wt. (g.)	Gain (g.)
9877	110	Dead		9872	87	64	-23
9894	66	Dead		9901	85	65	-20
9902	78	Dead		9909	86	70	-16
9932	94	110	16	9933	90	97	7
9869	112	Dead		9876	94	Dead	
9881	63	Dead		9882	58	54	-4
9905	76	Dead		9915	64	60	-4
9922	136	108	-28	9926	116	90	-26
9929	98	85	-13	9935	71	Dead	

2 I.U. Vitamin A alcohol				4 I.U.			
Rat no.	Initial wt. (g.)	4 weeks		Rat no.	Initial wt. (g.)	4 weeks	
		Wt. (g.)	Gain (g.)			Wt. (g.)	Gain (g.)
9885	51	66	15	9886	65	89	24
9910	94	106	12	9892	86	115	29
9919	81	93	12	9924	66	97	31
9939	66	82	16	9941	66	90	24
Mean ♂♂	73.0	86.8	13.8		70.8	97.8	27.0
9873	100	116	16	9874	73	84	11
9890	82	91	9	9898	64	76	12
9896	77	89	12	9906	74	87	13
9914	73	81	8	9928	96	105	9
9938	72	79	7	9937	65	80	15
Mean ♀♀	80.8	91.2	10.4		74.4	86.4	12.0
Mean ♂♂ and ♀♀	77.3	89.2	11.9		72.8	91.4	18.6

2 I.U. β-Carotene				4 I.U.			
Rat no.	Initial wt. (g.)	4 weeks		Rat no.	Initial wt. (g.)	4 weeks	
		Wt. (g.)	Gain (g.)			Wt. (g.)	Gain (g.)
9879	101	112	11	9880	98	124	26
9903	101	114	13	9884	67	85	18
9908	82	99	17	9895	100	139	39
9918	73	90	17	9917	82	106	24
Mean ♂♂	89.3	103.8	14.5		86.7	113.5	26.8
9868	110	110	0	9870	92	110	18
9883	63	70	7	9904	81	100	19
9889	82	103	21	9913	66	88	22
9897	96	109	13	9921	116	125	9
9920	127	139	12	9936	78	78	0
Mean ♀♀	95.6	106.2	10.6		86.6	100.2	13.6
Mean ♂♂ and ♀♀	92.8	105.1	12.3		86.7	106.1	19.4

2 I.U. Vitamin A ester				4 I.U.			
Rat no.	Initial wt. (g.)	4 weeks		Rat no.	Initial wt. (g.)	4 weeks	
		Wt. (g.)	Gain (g.)			Wt. (g.)	Gain (g.)
9887	66	74	8	9878	67	86	19
9893	74	86	12	9888	54	64	10
9900	70	88	18	9911	95	113	18
9934	107	131	24	9931	92	118	26
Mean ♂♂	79.3	94.8	15.5		77.0	95.3	18.3
9871	99	104	5	9867	111	121	10
9875	84	96	12	9899	80	105	25
9891	96	111	15	9912	70	96	26
9907	78	84	6	9923	85	106	21
9916	72	87	15	9930	108	113	5
Mean ♀♀	85.8	96.4	10.6		90.8	108.2	17.4
Mean ♂♂ and ♀♀	82.9	95.7	12.8		84.7	102.4	17.7

DISCUSSION

The results show without doubt that the pig, like the rat, is unable to make use of a hydroxy- β -ionone ring and cannot utilize zeaxanthin. Lund's [1938] results to the contrary were most probably due to active impurities present in his

preparation of zeaxanthin. It is possible that the slight difference between the behaviours of the rats receiving in our experiments the two levels of this carotenoid may have been due to traces of carotene or cryptoxanthin retained in our preparation in spite of the precautions taken to secure a pure specimen.

A striking result of the present work is the confirmation of the findings of Guilbert *et al.* [1937] that the pig utilizes carotene less efficiently than vitamin A even under conditions probably not far removed from optimal. The two pigs receiving 100 I.U. of β -carotene per 10 lb. live weight were unable to store any vitamin A in their livers. One of them died and the post-mortem findings were typical of vitamin A deficiency. The two pigs receiving 300 I.U. of β -carotene grew satisfactorily, but their liver reserves were slightly lower than those of pigs given only one-third of this quantity as preformed vitamin A. Making due allowance for the small number of pigs used in our experiments we feel it safe to suggest that for the pig the efficiency of conversion of β -carotene into vitamin A is at best no greater than 30–40%. On the other hand previously depleted rats utilized in our experiment β -carotene as efficiently as preformed vitamin A. The dosages were comparable with those received by the pigs, as 2 and 4 I.U. daily corresponded in this case to about 100 and 200 I.U./10 lb. live weight (Table 4). Though the treatment was preventive for pigs and curative for rats, both species were receiving similar levels of the provitamin dissolved in the same oil and the diets were also similar in being low in fat. It would seem therefore that the difference is a true one in metabolic behaviour.

The findings of Guilbert and his colleagues [Hart, 1940] point in the same direction and there are also indications that carnivora utilize carotene inefficiently as a source of vitamin A [Ahmad, 1931; Rea & Drummond, 1932; Frohring, 1935; cf., however, Bradfield & Smith, 1938]. Whatever the species, the ratio of the respective efficacies of vitamin A and carotene widens as the dose is increased [Guilbert *et al.* 1937; 1940]. Davies & Moore [1934] have reported that even the rat assimilates vitamin A at high levels much better than carotene. The careful tests of Wagner [1940] show the difference in the utilization of the provitamin and of vitamin A itself by man even after vitamin A deprivation. His findings are supported by numerous other reports, of which those of Booher *et al.* [1939], Booher & Calliston [1939] and Kreula & Virtanen [1939] may be quoted as examples. All these observations certainly bring out the complications and difficulties inherent in the use of carotene as a standard for vitamin A and make the suggestion of Guilbert *et al.* [1940] that separate standards for vitamin A and for carotene should be adopted worthy of serious consideration.

If the definition put forward by Guilbert *et al.* [1937] that the minimum level of vitamin A is one producing good growth but little storage in the liver is accepted, then our results are in good agreement with their findings. In our case the conditions were satisfied by 7.7 μ g. of vitamin A as the alcohol or its natural esters and 40 μ g. of β -carotene daily per kg. body weight. Figures in Table 1 show that the solutions of vitamin A alcohol and vitamin A ester deteriorated slightly during the tests. The loss of potency was of the order of 5% and if taken into consideration would reduce the estimate of the minimum requirements to 7.3 μ g. The figures given by the American workers for vitamin A and carotene were respectively 5.1–6.7 μ g. and 25–39 μ g.

The results of the rat assay of the vitamin A esters and vitamin A alcohol reported by us confirm the conclusions reached independently by Underhill [unpublished work] that there is no difference in the effect on the growth of vitamin A-deficient rats of equal quantities of vitamin A alcohol or natural vitamin A esters, when these quantities are estimated by measurement of the

spectrographic absorption at 328 $m\mu$. The significance of this result will be fully discussed by Underhill, so that it is only necessary to point out here that its attainment was probably due to the freedom of the preparations tested from oxidation products and other impurities which interfere with the spectrographic estimation.

The evidence as to the relative efficacies of free and esterified vitamin A for pigs fully supports the results obtained on rats, but owing to the use of fewer animals it carries less weight. There seems nevertheless to be some justification for the hypothesis that the two forms of the vitamin are equally, or nearly equally, effective for pigs.

SUMMARY

1. The vitamin A activities of carefully purified preparations of zeaxanthin, vitamin A alcohol, vitamin A ester and β -carotene were assayed on pigs and rats.
2. Zeaxanthin was biologically inactive for both species.
3. Pigs made less efficient use of β -carotene than rats and required, to cover their minimal requirements, about 300 I.U. of β -carotene or 100 I.U. of preformed vitamin A daily per 10 lb. live weight.
4. No difference was found in the biological activities of vitamin A alcohol and vitamin A natural ester for either pigs or rats.

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