LXXII. CRYSTALLINE ESTERS OF VITAMIN A

I. PREPARATION AND PROPERTIES

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PART I

THE preparation of the crystalline 2-naphthoate and anthraquinone-2-carboxylate of vitamin A has been reported by Hamano [1935; 1937] who used as starting material the non-saponifiable matter from fish liver oils which are exceptionally rich in this vitamin. Hamano found that these esters were biologically active but did not submit them to a quantitative animal assay. The work described in this paper was undertaken in order to find out whether crystalline esters could be prepared from vitamin A obtained from commercial oils and to compare their biological activity with that of the International Standard.

By molecular distillation of vitamin A alcohol, fractions with coefficients of absorption ($E_{1\text{cm}}^{1\%}$ 328 $m\mu$) exceeding 1600 were obtained. These were used for the preparation of several synthetic esters of which the 2-naphthoate and anthraquinone-2-carboxylate alone were obtained crystalline. The properties of the crystalline esters made in this way resembled those of the specimens prepared by Hamano, the anthraquinone-2-carboxylate being obtained in both the yellow and the red forms described by this author.

The ultraviolet absorption of the esters has been examined and from the results it has been deduced that the vitamin A alcohol combined in these substances has $E_{1\text{cm}}^{1\%}$ 328 $m\mu$ = 1800 approximately. Preliminary biological tests on the crystalline esters have been carried out by Dr Katharine Coward at the laboratories of the Pharmaceutical Society of Great Britain and by Dr S. W. F. Underhill in the physiological laboratories of the British Drug Houses Ltd. Both substances were found to be highly active and to have similar potencies when these were calculated on the basis of vitamin A content.

The acetate, diphenylacetate, 4-nitrobenzoate and stearate of vitamin A have also been prepared but have not as yet been obtained crystalline.

It was found that distilled vitamin A alcohol having $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu = 1600^1$ could be crystallized from methyl alcohol at low temperatures. The product obtained in this way had properties identical with those of crystalline vitamin A which had been prepared by the method of Holmes and Corbet [1937]. When working with our raw materials, distillation, followed by direct crystallization, proved to be the less tedious procedure. Two batches prepared by the latter method had $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu = 1800$ in ethyl alcohol and Carr-Price values 92,000 and 94,000.

The stability of the synthetic esters is of importance in connexion with the possibility of their use as biological standards. All the highly purified preparations of vitamin A and its esters which are described in this paper appear to deteriorate on storage in the absence of oxygen. The progress of this change is marked by a decrease in $E_{1\text{ cm.}}^{1\%} 328\text{ m}\mu$ and in Carr-Price value and the rate at which it takes place varies considerably; the crystalline 2-naphthoate and anthraquinone-2-carboxylate were found to be the most stable preparations of high potency, although the specimens of which portions were used for the biological tests deteriorated slightly during three months' storage *in vacuo*; solutions of these esters in arachis oil, however, appear to be quite stable. Crystalline vitamin A alcohol (on the other hand) appears to be more labile; Holmes and Corbet observed that the ultraviolet absorption of solutions of the crystals decreased very rapidly and a similar falling off was observed by us with a crystalline specimen during storage *in vacuo* at a temperature below its melting point.

EXPERIMENTAL

The purification of vitamin A by molecular distillation

Concentrates of vitamin A, substantially free from sterols, were prepared from fish liver oils in the usual manner. These were further purified by repeated fractional molecular distillation in a cyclic still of the type described by Hickman [1937]. In this apparatus, the distilland may be re-circulated through the still for any desired number of times. The rate of circulation may be varied at will, but a speed of about 6 ml. per min. was found to give the best results. Each passage of the distilland over the hot distillation surface at a temperature which may be predetermined constitutes a cycle. The distillation surface, consisting of a vertical metal cylinder, is placed co-axially within an air-cooled glass tube which acts as the condenser. The space between the hot surface and the condenser is about 1 cm. wide, and is evacuated to $1\text{--}3\mu$ by a condensation pump with a high pumping speed. The distillate flows down the condenser into a receiver which may be changed without interrupting the distillation. In using this apparatus for the distillation of viscous vitamin A concentrates, the latter were diluted with "residue oil" which was prepared from cod liver oil by removal of material volatilizing at or below 250° in a molecular still. Many distillations

¹ Smith *et al.* [1938] and Gillam & El Ridi [1938] have shown that the ultraviolet absorption of vitamin A preparations varies with the solvent used for the determination, while Holmes & Corbet have reported that Darby noticed a rapid decrease in the extinction coefficient of solutions of the crystalline vitamin. It is on account of these observations that we give the following particulars of the determination of the extinction coefficients quoted in this paper. Pure *isopropyl* alcohol was used as solvent in all cases except for the measurements on crystalline A when ethyl alcohol was employed. The determinations were made within 20–30 min. of the preparation of the solution and the latter was subjected for about 4 min. to the action of the light from a spark between electrodes of tungsten steel.

and redistillations were carried out during the preparation of the highly purified vitamin used in the experiments described in this paper. The results of two distillations are given in Table I.

Table I. *Distillation of crude vitamin A concentrate*

Fraction	Temperature ° C.	Pressure μ	No. of cycles	Wt. g.	Carr-Price value	Recovery %
Distilland: Run 7. Vitamin A concentrate, Carr-Price value 40,000						80 g.
"Residue oil"	...	...	...	...	...	120 g.
1	95	4-7	5	6.2	64,000	12
2	110	1-3	5	20.7	66,000	42
3	120	1-2	4	14.8	62,500	29
4	130	1-2	2	6.7	56,000	11
Total recovery						94
Run 20. Mixed fractions from previous distillations, Carr-Price value 53,750						77 g.
"Residue oil"	...	...	...	...	...	20 g.
1	85	2-3	3	6.9	67,500	10
2	95	1-2	5	11.5	80,000	22
3	105	1-2	5	17.2	68,750	29
4	115	1-2	3	11.5	66,250	18
5	125	1-2	7	10.3	55,000	14
Total recovery						93

Fraction 2 (run 20) had $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1690 (in alcohol). The purest fraction which we have obtained by molecular distillation had $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1950.

Crystallization of distilled vitamin A alcohol

A solution of vitamin A (20.4 g., Carr-Price value 81,000) in methyl alcohol (250 ml.) was cooled in solid carbon dioxide, inoculated with crystals obtained from a previous preparation, and allowed to warm up until only a small amount of solid remained undissolved. The bottle containing the solution was then covered with cotton wool and placed in a tin surrounded with solid carbon dioxide. After two days' storage at about -75° , the pale yellow crystals were collected on a sintered glass filter, cooled with solid carbon dioxide. The material was pressed on a porous plate and dried during 3 hr. in a high vacuum, at a temperature which was allowed to rise slowly from -8 to 4° . Yield 9.2 g.

This material when allowed to warm up slowly in an evacuated tube, melts at about 8° to a liquid which evolves vapour which may prove to be solvent of crystallization and simultaneously becomes more viscous. When completely freed from solvent in this manner, the product had Carr-Price value 92,000 and $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1820 (in ethyl alcohol).

Vitamin A stearate

Vitamin A alcohol (17.4 g., Carr-Price value 80,000) in ether, was treated with ethereal solutions of recrystallized stearyl chloride (16.2 g.), and triethylamine (17.4 g.). The solutions of the latter reagents were added in parallel portions and the reaction was carried out at 0° , and in an atmosphere of nitrogen. The mixture stood overnight at room temperature and was filtered to remove triethylamine hydrochloride. The filtrate was washed with water, aqueous NaHCO_3 and water, and the ether was evaporated in nitrogen. In order to remove any unchanged vitamin A alcohol, the residue remaining after evaporation of the ether was dissolved in light petroleum (125 ml.), and the solution was allowed to percolate

through a column of activated alumina (150 g.). This material adsorbs the free vitamin A but not the esters. The column was rinsed with light petroleum and the filtrate and rinsings were evaporated under diminished pressure. The residue (17.3 g., Carr-Price value 48,000) consisted of a pale yellow oil which solidified to a wax at about 17°. This material was dissolved in ether and the solution was cooled in solid carbon dioxide; the pale yellow solid ester was collected and dried at a low temperature. The Carr-Price value of the product fell from 45,000 to 30,000 in 48 hr., and the experiment was therefore not pursued further.

Vitamin A diphenylacetate

Vitamin A (14 g., Carr-Price value 78,000) in pyridine (25 ml.) was treated with a solution of diphenylacetyl chloride (10 g.) in benzene (40 ml.). The mixture was warmed on the water bath for 60 min., then cooled and diluted with ethyl acetate. The solution was washed, dried and evaporated. The residue was dissolved in light petroleum and the solution was extracted ten times with an equal volume of 95 % methyl alcohol in order to remove unchanged vitamin A. The ester (4.9 g.) which remained after evaporation of the light petroleum had Carr-Price value 37,500, but could not be crystallized.

Vitamin A 4-nitrobenzoate

This ester, which was prepared in the usual manner by interaction between vitamin A and 4-nitrobenzoyl chloride in pyridine, formed a red oil which was soluble in ethyl acetate, acetone and ether, sparingly soluble in light petroleum, and insoluble in methyl alcohol. It was not obtained crystalline.

Vitamin A acetate

This compound, which was first described by Karrer [1931], was prepared by the interaction of distilled vitamin A (Carr-Price value 50,000) and redistilled acetyl chloride in pyridine. Since vitamin A and its acetate distil at approximately equal rates at the same temperature, the crude product was partitioned between methyl alcohol and light petroleum, in order to remove any vitamin that might have escaped esterification. For this, a solution of the acetate in light petroleum was extracted eight times with an equal volume of 95 % methyl alcohol. The acetate is somewhat soluble in methyl alcohol, and the process involved considerable loss. The acetate which was recovered from the petroleum layer had a Carr-Price value 34,000. It was diluted with residue oil and distilled. The most potent fraction distilled at 100° and consisted of a pale yellow, mobile oil with Carr-Price value 55,000 and $E_{1\text{cm}}^{1\%}$ 328 $m\mu$ = 860.

Vitamin A anthraquinone-2-carboxylate

For the preparation of this ester, and of the 2-naphthoate, the method described by Hamano was used. The details are given in view of the difference in our starting material.

Distilled vitamin A alcohol (6.1 g., Carr-Price value 80,000) in pyridine (20 ml.) was treated with a solution of recrystallized anthraquinone-2-carboxyl chloride (4.3 g., M.P. 150°) in warm benzene (40 ml.). The mixture was heated on the water bath for 30 min. in an atmosphere of nitrogen, and the greater part of the solvents was then removed in a stream of this gas. The residue was dissolved in warm ethyl acetate (200 ml.). The solution was filtered and washed with water, dilute acid, water and aqueous NaHCO_3 . After filtration to remove suspended sodium anthraquinone-2-carboxylate, and a final wash with water, the solution was dried (Na_2SO_4) and evaporated under diminished pressure. The

residue was dissolved in a small volume of acetone from which the ester soon began to separate in the form of small yellow plates. After two further crystallizations from acetone, the product formed macroscopic plates or prisms which melted at 126° (corr.) after turning red at about 123°. Hamano gives 124° as the melting point of the yellow modification of this ester. The Carr-Price value was 47,000 and the coefficient of absorption $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 938. These figures correspond to Carr-Price value 85,000 and $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1510¹ for vitamin A alcohol. (Found C. 80.4, 80.7 %; H. 6.97, 6.81 %; $C_{35}H_{36}O_4$ requires C. 80.8 %, H. 6.92 %.)

The anthraquinone-2-carboxylate has also been obtained in the red form described by Hamano. This form was found to be less stable and more soluble at room temperature than the yellow allotrope. Conversion of the red into the yellow allotrope may be effected by solution in hot acetone, followed, after cooling, by inoculation of the solution with a crystal of the appropriate modification. After several recrystallizations from ethyl acetate-alcohol, the red modification formed long flat prisms having blunt ends, M.P. 118–120°. The Carr-Price value was 47,000 and the coefficient of absorption $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1090. These figures correspond to Carr-Price value 85,000 and $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1790¹ for vitamin A alcohol.

Vitamin A-2-naphthoate

Vitamin A alcohol (17.8 g., Carr-Price value 80,000) in pyridine (30 ml.) was treated in an atmosphere of nitrogen with 2-naphthoyl chloride (8.2 g.) in benzene (40 ml.). The mixture was heated during 30 min. on a water bath and the greater part of the solvents was then distilled off in a stream of nitrogen. The residue was taken up in ether. Further working as described for the anthraquinone-carboxylate gave a pale yellow syrup which was dissolved in acetone (ca. 30 ml.). The solution was cooled to about –75° as described by Hamano; the ester could not be induced to crystallize after several days at this temperature, and the solution was therefore treated with methyl alcohol (ca. 10 ml.). Crystallization started at once and was completed by cooling to –75°. The precipitate, which was partly crystalline and partly gummy, was collected and recrystallized from a small volume of acetone in which the ester is rather soluble. After four crystallizations from ethyl alcohol, the product weighed 950 mg. and melted at 78° (corr.). Hamano gives 76° as the melting point of vitamin A 2-naphthoate.

The ester crystallized from alcohol as large pale yellow plates, and from acetone as pale yellow elongated prisms. The specimen used for the biological test (Part II) had, immediately after its preparation: Carr-Price value 50,000 and $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1180 corresponding to Carr-Price value 76,900 and $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1790² for the vitamin A alcohol. (Found: C, 83.9, 83.7 %; H, 8.06, 8.18 %. $C_{31}H_{36}O_2$ requires C, 84.5 %; H, 8.18 %.)

Another specimen had $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1190 and Carr-Price value 62,500. These values correspond to $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ = 1800² and Carr-Price value 96,000 for vitamin A.

The effect of storage on the properties of the crystalline esters

Samples of the batches of the anthraquinone-2-carboxylate and 2-naphthoate which were used for animal assays were stored in evacuated amber tubes. After about 3 months the esters were unaltered in appearance and had undergone no

¹ These values have been corrected for the absorption due to anthraquinone-2-carboxylic acid; ethyl anthraquinone-2-carboxylate was found to have ϵ_{328} = 5600.

² These values have been corrected for the ultraviolet absorption of 2-naphthoic acid for which we found ϵ_{328} = 820.

marked change in melting point. Further examination, however, disclosed that the ultraviolet absorption of both esters had decreased by about 20 % and that whereas the Carr-Price value of the naphthoate was practically unchanged, that of the anthraquinone carboxylate had decreased by about 15 %. This behaviour contrasted sharply with the apparent stability of the solutions of these esters in arachis oil, which were used for the biological tests. The effects of storage *in vacuo* are summarized in Table II. Exposure of the crystals to the air brought about rapid decomposition.

Table II

Ester	...	Anthraquinone-2-carboxylate		2-Naphthoate	
		5. iii. 38	13. vi. 38	17. iii. 38	13. iii. 38
Date	...				
$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$		938	750	1180	980
Carr-Price value		47,000	40,000	50,000	52,000
M.P. (corr.)		126° after turning red at 123°	127° after turning red at 121°	78°	77-79°

PART II

Biological tests were carried out on the yellow modification of vitamin A anthraquinone-2-carboxylate and on vitamin A 2-naphthoate. Both esters were dissolved in arachis oil containing 0.01 % quinol, the anthraquinone-2-carboxylate at a concentration of 0.15 % (solution P. 87) and the 2-naphthoate at 0.2 % (solution P. 90). The solutions were kept under nitrogen in the refrigerator, dilutions being prepared at regular intervals for administration to the animals: the diluted solutions were also kept under the same conditions except when required for feeding. The coefficients of absorption and the Carr-Price blue values of the two solutions (P. 87 and P. 90) were determined at intervals during the course of the experiments (Table III): the results show that no deterioration occurred during this period. (Later determinations of the blue values have shown that the solutions have remained stable for 7 months.)

The biological tests were carried out by us independently, employing the technique customary in each laboratory.

Table III

P. 87			P. 90		
Date	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$	Carr-Price blue value	Date	$E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$	Carr-Price blue value
16. iii. 38	—	64	22. iii. 38	2.14	100
17. iii. 38	1.42	64	28. iii. 38	2.15	100
21. iii. 38	1.43	64	11. iv. 38	2.14	96
11. iv. 38	1.51	60	26. iv. 38	2.10	96
26. iv. 38	1.55	60	14. vi. 38	2.03	100
14. vi. 38	1.55	60			

I. Determination of biological potency by S. W. F. U.

For the feeding experiments the solutions of the esters were further diluted with arachis oil containing quinol. P. 87 was diluted 1 : 6 and 1 : 12 and P. 90 1 : 10 and 1 : 20, that is, in both cases to make solutions with Carr-Price blue values of 10 and 5 respectively. Solutions containing 200 and 100 units per g. were similarly prepared from the solution of the international standard β -carotene. The dilutions were checked by determination of the blue value.

The biological tests were carried out on young rats which were given our vitamin A-free diet [Culhane, 1933] when they had reached a weight of about 40 g. each. When the weights became stationary the animals were divided into groups, those from different litters being distributed as evenly as possible amongst the different groups. All the groups received the dose of 0.02 g. of oil twice a week given by calibrated pipette directly into the animals' mouths. Three groups of each sex were given doses of the two esters and of the standard respectively. Another three groups of each sex were given a dose of ester or standard once a week only, alternately with a dose of arachis oil. The seventh group (negative controls) were given only doses of arachis oil twice a week.

Two experiments were carried out; in the first 78 animals were employed—51 bucks and 27 does; of these 8 bucks and 3 does were kept as negative controls. The doses were 0.02 g. of the solutions of the esters diluted 1 : 6 (P. 87) and 1 : 10 (P. 90) and four units of standard, given once and twice weekly. In the second experiment 57 animals were used—26 bucks and 36 does; 3 bucks and 4 does were kept as negative controls. The doses of the esters were 0.02 g. of the dilutions 1 : 12 (P. 87) and 1 : 20 (P. 90) with two units of standard, given once and twice weekly.

The average growth rate of the different groups in g. per week was calculated after the supplements had been given for three weeks. The results are given in Table IV.

Table IV

Exp. no.	Weekly dose	Dilution employed	♂		♀	
			No. of rats	Growth in g. per week	No. of rats	Growth in g. per week
Vitamin A anthraquinone-2-carboxylate: P. 87						
1	0.04 g.	1 : 6	4	9.8	5	7.5
	0.02 g.	1 : 6	4	7.0	6	5.0
2	0.04 g.	1 : 12	2	5.5	4	5.4
	0.02 g.	1 : 12	3	4.4	5	1.4
Vitamin A 2-naphthoate: P. 90						
1	0.04 g.	1 : 10	4	10.9	6	7.9
	0.02 g.	1 : 10	7	9.1	4	5.6
2	0.04 g.	1 : 20	3	4.3	4	5.3
	0.02 g.	1 : 20	4	2.3	4	1.8
International standard						
1	8 units		8	8.8	7	6.0
	4 "		6	7.6	6	3.2
2	4 "		6	1.4	5	1.7
	2 "		3	-0.7	7	-1.5

Since standard dose-response curves for bucks and does are not in use in this laboratory, curves relating the increase in weight to the logarithm of the dose were first calculated for each solution P. 87 and P. 90 and for the international standard. The average slope of the three curves was next obtained, making allowance for the number of animals used, and a curve with this slope adjusted (mathematically) to the midpoint of each of the individual curves, so that the ratios of the doses of P. 87 and P. 90 to the dose of standard could be found. From these figures the potency of each solution was calculated, the results given by the bucks and does being averaged, with weighting for the number of animals used. The mean potencies are given in Table V.

Table V. *Potency of solutions P. 87 and P. 90 in units per g.*

Exp. no.	P. 87	P. 90
1	1580	3830
2	4250	4880
Mean	2740	4280
Standard error	+35 % -26 %	+36 % -27 %

In the first experiment the average growth rate was also determined for a period of 5 weeks: the potencies of the solutions calculated from these figures are not significantly different from those given in Table V.

The potencies of the esters and of vitamin A alcohol were calculated from the mean potencies of the solutions P. 87 and P. 90. The potency of the anthraquinone-2-carboxylate is 1,827,000 units per g. and of the 2-naphthoate 2,140,000 units per g. The figures for the potency of vitamin A alcohol are, respectively, 3,320,000 and 3,293,000 units per g. Since β -carotene contains, by definition, 1,667,000 units of vitamin A activity per g., vitamin A is, weight for weight, twice as potent as β -carotene.

II. *Determination of biological potency by K. H. C.*

The vitamin A potency of the two preparations was determined by the increase in weight brought about by giving doses to rats which had been fed on a vitamin A-free diet until they had become steady in weight. The details of the method have been fully described elsewhere [Coward, 1938].

The amount of vitamin A in P. 87 was theoretically 0.0825 % (mol. wt. vitamin A anthraquinone-2-carboxylate 520: mol. wt. vitamin A alcohol 286). The dose of solution tested was 0.5 mg. (diluted with arachis oil 1 : 20) per day in comparison with one unit of the international standard in 10 mg. arachis oil.

The amount of vitamin A in P. 90 was theoretically 0.13 % (mol. wt. vitamin A 2-naphthoate 440). The dose of solution tested was 0.33 mg. (diluted with arachis oil 1 : 30) per day in comparison with 0.5 mg. of the first solution and one unit of the international standard in 10 mg. arachis oil.

The results were calculated in the way in general use in the laboratories of the Pharmaceutical Society. They are summarized in Table VI.

Table VI

	Dose	No. of rats		Increase in wt.		Corresponding abscissae on curves of response		Weighted mean of abscissae
		♂	♀	♂ g.	♀ g.	♂	♀	
P. 87	0.5 mg.	5	9	13.0	15.3	1.72	2.48	2.21
P. 90	0.33 mg.	5	7	26.6	13.4	4.94	1.94	3.19
Vit. A	1 I.U.	4	10	22.5	10.0	3.60	1.25	1.92

$$\text{P. 87.} \quad \frac{\text{The vitamin A content of 0.5 mg. P. 87}}{1 \text{ I.U. of vitamin A}} = \frac{2.21}{1.92} = 1.15.$$

Therefore 0.5 mg. P. 87 contains 1.15 units vitamin A and the solution contains 2300 I.U. of vitamin A per g.

$$\text{P. 90.} \quad \frac{\text{The vitamin A content of 0.33 mg. P. 90}}{1 \text{ I.U. of vitamin A}} = \frac{3.19}{1.92} = 1.66.$$

Therefore, 0.33 mg. P. 90 contains 1.66 units vitamin A and the solution contains 5000 I.U. of vitamin A per g.

The potency of vitamin A according to the first test:

$$\text{Dose of vitamin A given was } 0.0005 \times \frac{0.0825}{100} \text{ g.} = 0.0000004125 \text{ g.} \\ = 0.4125 \mu\text{g.}$$

$$\frac{0.4125 \mu\text{g. vitamin A}}{0.6 \mu\text{g. } \beta\text{-carotene}} = \frac{2.21}{1.92}$$

Therefore vitamin A is 1.67 times as potent as β -carotene.

The potency of vitamin A according to the second test:

$$\text{Dose of vitamin A given was } 0.00033 \times \frac{0.13}{100} \text{ g.} = 0.000000429 \text{ g.} \\ = 0.429 \mu\text{g.}$$

$$\text{Therefore } \frac{0.429 \mu\text{g. vitamin A}}{0.6 \mu\text{g. } \beta\text{-carotene}} = \frac{3.19}{1.92}$$

Therefore vitamin A is 2.32 times as potent as β -carotene.

The average of these two figures gives the potency of vitamin A as twice that of β -carotene, weight for weight.

DISCUSSION

We consider that our results, which were obtained independently in different laboratories, show reasonably good agreement: they are summarized in Table VII.

Table VII

	P. 87 Vitamin A anthraquinone 2-carboxylate	P. 90 Vitamin A 2-naphthoate
Concentration of solution in arachis oil	0.15 %	0.2 %
$E_{1\text{ cm}}^{1\%}$, 328 m μ	1.49	2.11
Carr-Price blue value	60	100
Potency: Units per g. S. W. F. U. 1	1580	3830
S. W. F. U. 2	4250	4880
K. H. C.	2300	5000
Mean	2625	4450
"Conversion factor" (corr.)	1920	2150

The potencies of the esters and of vitamin A alcohol calculated from these figures are:

	Units per g.		Units per g.
Vitamin A anthraquinone-2-carboxylate	1,750,000	Vitamin A alcohol	3,181,000
Vitamin A 2-naphthoate	2,225,000	Vitamin A alcohol	3,424,000

In the case of each ester the calculated potency of the vitamin A alcohol is approximately twice that of β -carotene.

For some years it has been assumed that the molecule of β -carotene breaks up in the animal body and by the addition of an OH group and an H atom to each of the broken ends, two new molecules are formed, each of which is a molecule of vitamin A. Thus 536 g. of β -carotene would give 572 g. of vitamin A and vitamin A and β -carotene would have very nearly equal activities weight for weight. But the two samples of vitamin A examined biologically have been shown to have 1.91 and 2.05 times respectively the vitamin A potency of β -carotene. (The difference between these figures is not significant.) This is in agreement with the results of Holmes & Corbet [1937] who found the potency of their vitamin A to be 3,000,000 i.u. per g. These results indicate either that (a) β -carotene is not converted quantitatively into vitamin A in the animal body or that (b) the molecule

of β -carotene gives rise to only one molecule of vitamin A, the other part of the molecule having no activity either when it was part of the carotene molecule or when it was separated from it. The latter possibility seems to be the more probable one since otherwise it is unlikely that the several tests should all have indicated that vitamin A has double the potency of β -carotene, weight for weight. Also it might be expected that, if β -carotene is not converted quantitatively into vitamin A in the body, the variation in different rats' powers of conversion would result in a greater variation in response to a given dose of carotene than that in response to a dose of any other form of vitamin A. Coward [1936] has shown that the standard deviation of the response of rats which had been given vitamin A in the form of carotene in vegetables was not greater than, and was apparently a little less than, that of rats given true vitamin A in cod liver oil, butters and vitaminized margarine (Table VIII).

Table VIII

	No. of rats from which the calculation was made		Standard deviation	
	♂	♀	♂	♀
1. Tests on a cod liver oil, Z	510	645	11.58	9.51
2. Tests on (a) butters and	257	315	12.75	9.72
(b) margarines	281	371	11.45	10.03
1 and 2 together	1048	1331	11.85	9.71
*Tests on β -carotene, the international standard	207	243	8.95	7.84

* Not before published.

A calculation has since been made of the standard deviation of the response of rats to doses of the international standard (i.e. β -carotene in oil) (Table VIII). It was found to be less than that of rats given doses of vitamin A in any other form and the difference was almost certainly significant.

$\frac{\sigma_1 - \sigma_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$, in which σ is the standard deviation of a single observation of increase in weight, and $\epsilon = \frac{\sigma}{\sqrt{2(n-m)}}$, n being the number of rats and m the number of groups of rats, was 5.06 for the male rats and 4.23 for the female rats. Thus the variation in response to doses of β -carotene in oil is less than the variation in response to doses of true vitamin A in oil, and therefore the β -carotene is probably not incompletely converted into vitamin A in the animal body.

It must be concluded, therefore, that the molecule of β -carotene has only one active group and that, on breaking down in the animal body, only one molecule of vitamin A is formed from one molecule of β -carotene, the other part of the carotene molecule presumably being converted into some substance which is without any vitamin A activity.

The fact that the calculated potency of vitamin A is the same in the case of each ester shows that the vitamin in each is assimilated by the rat to the same extent: it is of interest that esters which are not natural to the body can be apparently completely utilized. Our results taken in conjunction with those of Holmes & Corbet afford strong evidence that the rat utilizes indifferently and probably completely vitamin A and its natural esters. Utilization of the synthetic esters presumably depends upon their hydrolysis in the body: it will be of interest if a non-hydrolysable and, therefore, inactive ester is prepared in the future.

It has been stated [Wilkinson, 1938] that the curve of response for vitamin A is different from that for carotene. This can be tested for these experiments by comparing the increases in weight with the pairs of doses of esters and international standard in Table IV, since the slope of the curve for each pair of doses is formed by dividing the difference between the increases in weight by the difference between the logarithms of the doses given, which in these experiments is always 0.301. For the male rats, the differences between the increases in weight from pairs of doses of the esters were 2.8, 1.1, 1.8 and 2.0 and for the female rats, 2.5, 4.0, 2.3, 3.5; the corresponding figures from pairs of doses of the standard were for the male rats 1.2 and 2.1, and for the female rats 2.8 and 3.2. It is obvious, without further calculation, that the slopes of the curves of response for vitamin A are, under the conditions of this experiment at least, not different from those for β -carotene. Since pairs of doses were not given in the experiment recorded in Table VI, it is not possible to test these figures in the same way.

We have also calculated from our figures the value of the "conversion factor" for converting the value of the extinction coefficient of vitamin A into biological units per g. For this purpose it is necessary to correct the observed $E_{1\text{ cm}}^{1\%}$ 328 $m\mu$ values (Table III) for the absorption due to the acid fraction of the ester molecules. The corrections are for P. 87, 0.12, and for P. 90, 0.04, and the observed values must be reduced by these amounts. The value of the conversion factor for the vitamin A anthraquinone-2-carboxylate solution is then 1920 and for the solution of the vitamin A 2-naphthoate, 2150: the difference between these figures is not significant. The factor accepted for the B.P. addendum 1936 is 1600, but our results are within the upper range of the figures from which the average of 1600 was obtained. They suggest, however, that the true value for the "conversion factor" for pure vitamin A is about 2000. Two explanations may be offered for the variations from this figure which have frequently been observed; either the solution examined has contained material which absorbs at wave lengths in the region of 328 $m\mu$, but is not vitamin A or vitamin A in the form in which it is usually found, or the absorption of the vitamin from the intestine, or its utilization in the body of the rat varies according to its source, or according to the diet on which the animals are maintained. Our own results incline us to favour the former explanation although they do not exclude the latter.

SUMMARY

Part I

1. Vitamin A has been obtained in a high state of purity by distillation in the Hickman cyclic still.
2. The distilled vitamin has been crystallized directly from methyl alcohol at a low temperature.
3. The stearate, 4-nitrobenzoate, diphenylacetate and acetate have been prepared from highly purified vitamin A, but have not been obtained crystalline.
4. The crystalline anthraquinone-2-carboxylate and 2-naphthoate of vitamin A have been prepared from material purified by molecular distillation. These esters were found to resemble the anthraquinone-2-carboxylate and 2-naphthoate prepared by Hamano from undistilled concentrates.

Part II

1. The biological potencies of two esters of vitamin A have been determined in terms of the international standard β -carotene in simultaneous tests.
2. Vitamin A anthraquinone-2-carboxylate was found to contain 1,750,000 units per g. and vitamin A 2-naphthoate 2,225,000 units per g.

3. The potency of vitamin A alcohol is therefore 3,181,000 I.U. per g. according to the tests on the first ester and 3,424,000 I.U. per g. according to the tests on the second ester.

4. Since, by definition, β -carotene contains 1,670,000 I.U. of vitamin A activity per g., vitamin A itself is twice as active as β -carotene, weight for weight.

5. The factor for converting the coefficient of absorption at 328 m μ into biological units per g. ("conversion factor") was found to be about 2000.

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