CXXXVII. ABSORPTION SPECTRA OF SUBSTANCES DERIVED FROM VITAMIN A.

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IN continuation of earlier work on vitamin A we have investigated the ultra-violet absorption of certain products obtained after the vitamin has been decomposed. At the same time, we have tried to elucidate the mechanism of the antimony trichloride colour test.

As is well known, the blue colour is transient, and for purposes of assay it is necessary to determine the intensity of colour during the first minute, as otherwise the rapid fading tends to vitiate the results. Little is known concerning the secondary reactions which occur, and even the primary process is not fully understood. For the full development of the blue colour, the reagent must be nearly saturated and, it would appear, the SbCl_3 /vitamin A ratio must be very high (order 20,000 : 1). This fact suggests that the blue substance (or substances) is an addition compound; the hypothesis of more than one coloured addition complex has already been advanced to account for the two apparently independent absorption bands seen in the blue solutions [Gillam and Morton, 1931; Heilbron, Gillam and Morton, 1931]. It is possible that the primary process, resulting in the blue colour, leaves the vitamin A molecule intact, whilst the secondary reactions associated with the fading of the colour bring about decomposition. To test this view, the blue solutions have been poured into large volumes of water so that the antimony is precipitated as oxychloride. The organic matter is then recovered and its properties studied.

The general method adopted is as follows. A known weight of liver oil or concentrate dissolved in chloroform is treated with a saturated solution of antimony trichloride in chloroform and the mixture shaken for one minute to allow the blue colour to develop fully. The solution is then poured into water in a separating funnel and shaken vigorously to complete the decomposition of the antimony trichloride. As it is impossible to separate the chloroform layer owing to the precipitated antimony oxychloride, the latter is brought into solution by careful addition of concentrated hydrochloric acid, when separation into two layers readily occurs. The chloroform extract is washed successively with sodium carbonate and water and dried over sodium sulphate. After removal of solvent, the residual oil (or concentrate) is very similar in appearance to the original material and in all cases gives a blue or

purple colour with antimony trichloride, similar to, but weaker than, that given by the original.

An examination of the ultra-violet absorption spectra of the solutions in chloroform reveals, however, as shown in Fig. 1, in place of the broad vitamin A

Fig. 1.

----- Cod-liver oil 1 % (1 cm.) alcohol.

— The same oil "regenerated" 1 % (1 cm.) chloroform.

Table I.

Material	$E_{1\text{cm.}}^{1\%}$	Blue colour with antimony trichloride		Wave-length max. $m\mu$	$E_{1\text{cm.}}^{1\%}$	Wave-length max. $m\mu$	Intensity of ultra-violet absorption $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$ gross value	Positions of maxima in the ultra-violet absorption spectrum (solutions in chloroform) wave-lengths of maxima in $m\mu$
		Wave-length max. $m\mu$	$E_{1\text{cm.}}^{1\%}$					
Cod-liver oil A	1.29	606	1.11	575	1.15			
Do. regenerated	0.96	615	0.77	575	0.72			—, 399, 376, 356, (340), (325), —, (279)
Cod-liver oil B	2.7	603	2.3	573	2.0			
Do. regenerated	1.16	616	1.05	580	1.1			—, 399, 377, 357, 342, 325, 308, (286)
Cod-liver oil C	2.5	604	1.92	572	1.88			
Do. regenerated	1.1	615	1.0	572	0.88			—, 398, 376, 357, 340, 324, 308, 280
Halibut oil	20.4	615	not examined		—			
Do. regenerated	4.6	613	not examined		4.75			—, 402, 378, 358, 340, (324), (309)
Old halibut non-sap.	66.5	616	55.4	583	58			
Do. regenerated	20.7	610	31.9	581	35			(423), 402, 377, 356, 340, (327). —, —
Cod-liver oil non-sap.	213	615	124	580	115			
Do. regenerated	—	no band	92	586	67			425, 400, 379, 357, (340), —, (310), (285)
Halibut concentrate	950	620	order 500	583	370			
Do. regenerated	—	no band	142	590	140			425, 402, 378, 357, (340), —, (309), —
Mammalian liver concentrate	56.5	610	37.4	583	23			
Do. regenerated	4.0	610	6.0	583	6.0			420, 399, 376, 357, 341, 326, 309, —
Cod-liver oil C regenerated in CHCl_3		blue colour not studied				—		—, 398, 375, 355, 341, 325, (308), —

band (max. 328 $m\mu$) a series of narrow bands with maxima near 425, 399, 376, 357, 340, 324, 308 and 280 $m\mu$. From the data given in Table I it will be seen that after vitamin A and antimony trichloride in chloroform have reacted to form the highest attainable proportion of blue pigment (about 1 minute), a part of the vitamin A can still be recovered unchanged, although an appreciable proportion has undergone decomposition. The narrow absorption bands shown by the new material make the reaction easy to follow with the spectroscope. The procedure described above has been applied to a variety of fish-liver oils, mammalian liver fats and a wide range of concentrates. The wave-lengths of maximum absorption are always the same, indicating that the decomposition is qualitatively independent of the concentration of vitamin A; the intensities of absorption are always of the same order as the intensity of the vitamin A band in the starting material. Although the 328 $m\mu$ band cannot be observed as such in the "regenerated" material, its presence, masked by the highly selective absorption of the new products, can be inferred with certainty from the general shape of the absorption curve in conjunction with the results obtained by applying the colour test to the recovered materials.

It is interesting to note that the maxima for the new products differ noticeably from those recorded previously for the acids obtained by the hydrolysis of liver oils and concentrates [Gillam, Heilbron *et al.*, 1931] particularly after allowing for solvent displacement of wave-lengths.

	λ max. in $m\mu$									
Regenerated products (in chloroform)	425,	399,	376,	357,	340,	324,	308,	280		
Cod-liver oil acids (in alcohol)		392,	375,	350,	330,	316,	302,	281,	269,	235

The similarity of the resolution into narrow bands may indicate a structural resemblance. That the "regenerated" products are not acidic is, however, shown by the fact that no substances showing narrow absorption bands are extracted on washing with either sodium carbonate or sodium hydroxide. Water is also not essential to the appearance of the narrow bands, since they can be recorded in solutions prepared simply by diluting the blue solutions with much chloroform, making no attempt to eliminate the antimony trichloride.

Other methods for obtaining the substances showing narrow bands.

A. When unsaponifiable extracts from liver oils and fats are dissolved in absolute alcohol containing a little dry hydrogen chloride, the ultra-violet absorption spectrum undergoes a considerable change. For example, a 0.005% solution of a rich halibut-liver oil concentrate, dissolved in absolute alcohol, was found to exhibit the normal vitamin A band, the intensity maximum occurring at $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$, 1100. The large absorption band was completely free from "fine" structure. When, however, the same concentrate in alcoholic hydrogen chloride was examined, it was found not only that $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ had fallen to 575, but the selective absorption had been displaced towards the visible

and a succession of narrow bands was recorded, the maxima being at 419, 392, 369, 350 and 333 $m\mu$. The appearance of this set of narrow absorption bands is shown perfectly clearly with even the richest concentrates, and the conclusion cannot be evaded that these bands are characteristic of a material which is either an isomeride of vitamin A or a product very easily derived from vitamin A.

In order to elucidate the mechanism of the action of hydrogen chloride on vitamin A, we have studied the effect of varying the proportions of vitamin A and acid. Using a 0.006 % solution of rich concentrate containing perhaps 50 % vitamin A, when the concentration of hydrogen chloride is low (order 10^{-3} – 10^{-4} N) the narrow bands appear slowly, approaching maximum definition in 1 to 2 days and thereafter gradually disappearing. With a higher concentration of hydrogen chloride ($> N/50$) the narrow bands are fully developed by the time (30 minutes) the spectral absorption can be photographed, but the rate of disappearance appears to be definitely increased. Assuming provisionally that the intensity of the 328 $m\mu$ band for "pure" vitamin A is $E_{1\text{cm.}}^{1\%} = 1350$, and that the molecular weight is near 300 [Heilbron *et al.*, 1932] it is possible to calculate the relative molecular concentrations of vitamin A and hydrogen chloride. On this basis the range over which our observations extend is $[\text{vitamin A}]/[\text{HCl}] = 1/8000$ to $1/0.4$. The degree to which the narrow bands can be developed is somewhat variable and there seems to be little doubt that, under the experimental conditions, the substance (or substances) responsible for these bands is somewhat unstable. Many of the differences observed can be readily accounted for by the superposition of the new absorption spectrum on that characteristic of the unchanged vitamin A. The main product, when prepared under favourable conditions, exhibits maxima at 392, 369, 350 and 333 $m\mu$ (see Fig. 2), the 369 $m\mu$ maximum being the most intense. This stage is never attained with very dilute HCl even when in excess over the vitamin, whilst unduly strong acid merely increases the instability of the new product.

The determining factor appears to be the absolute concentration of the HCl rather than the $[\text{vitamin A}]/[\text{HCl}]$ ratio, since, using the same strength of acid, no difference can be detected in the absorption spectrum whether this ratio is $1/3$ or $1/300$. On standing, the definition slowly decreases, but this tendency can be arrested, if not entirely avoided, by the addition of sufficient sodium ethoxide solution to neutralise the hydrogen chloride. Similar arrests can be obtained earlier or later in the reaction between vitamin A and hydrogen chloride. It should be remarked, however, that there is no evidence of reversibility, the sodium ethoxide merely tending to stabilise the condition of affairs at the moment it is added.

The best method for obtaining solvent-free material rich in the new absorbing compound is to dissolve 0.5–1.0 g. of a rich concentrate in 100 cc. alcohol and to add enough alcoholic hydrogen chloride to make the solution approximately $N/30$. The mixture is left to stand in the cold for 20 minutes.

Fig. 2 a.

Fig. 2 b.

Fig. 2 c.

Fig. 2 d.

Fig. 2 e.

Absorption curves illustrating successive stages in the action of alcoholic hydrogen chloride on vitamin A.

2 a. Vitamin A concentrate in abs. alcohol. (Vitamin content about 55 % of that of the richest preparation so far obtained.)

2 b. Vitamin A concentrate in alcoholic hydrogen chloride. This curve shows the earliest detectable change.

2 c. A later stage in which the alcoholic hydrogen chloride has caused the narrow bands to appear more clearly.

2 d. The absorption curve observed at the point at which the narrow bands reach their maximum definition. The curve drawn with a continuous line can be reproduced without difficulty and it should be noticed that the highest absorption occurs at 369 $m\mu$. The broken lines indicate that strictly reproducible results in this region of the spectrum are not always obtained.

2 e. The absorption curve obtained with vitamin A and alcoholic hydrogen chloride after the mixture (*cf.* 2 d) had been left to stand for some days. The poorer definition is worthy of note.

It is then poured into water (400 cc.) and the yellow emulsion extracted several times with small quantities of low-boiling petroleum. After washing with water, and drying over sodium sulphate, the petroleum extract exhibits well developed narrow bands at 392, 369 (highest), 350, and 333 $m\mu$, and ill-defined bands in the regions 316–320, 286–290, 272, 261 and 254 $m\mu$ (these ill-defined maxima are recorded because they have been repeatedly observed, but at the present stage of the enquiry they are much less important than the group 393, 369, 350, 333 $m\mu$). The viscous red-brown oil remaining after removal of solvent exhibits the usual four main bands with great clarity. In an attempt to gain some insight into the mechanism of the reaction leading to this new material, molecular weight ~~determinations~~, micro-analyses and colour test determinations were carried out and are summarised in Table II.

Table II.

	Vitamin A concentrate	New product
Colour test	$E_{1\text{cm.}}^{1\%}$ 617 $m\mu$ 3900 580 $m\mu$ 2300	$E_{1\text{cm.}}^{1\%}$ 617 $m\mu$ 2350 580 $m\mu$ 1400
Micro-analysis	C, 82.8 % H, 10.9 %	C, 82.9, 82.7 % H, 10.4, 10.6 %
Molecular weight (cryoscopic method: solvent benzene)	327	403

The results show that the new product is heterogeneous and that [Heilbron *et al.*, 1932] some polymerisation has probably occurred. The blue value is somewhat higher than would be expected from the ultra-violet absorption.

B. The appearance of narrow bands during the slow high vacuum distillation of rich concentrates is discussed elsewhere. In order to discover how far this effect was due to the prolonged heating, a very rich vitamin A preparation (0.3 g.) was heated in an atmosphere of nitrogen, the temperature being maintained within the limits 120–125°. Small weighed portions were removed from time to time and studied spectroscopically. The results may be summarised as follows. The starting material exhibited the vitamin A band at $E_{1\text{cm.}}^{1\%}$ 328 $m\mu$, 1100, the absorption curve showing great persistence, as will be realised from the fact that the minimum at 245–250 $m\mu$ occurred at $E_{1\text{cm.}}^{1\%}$ 300. After 6 hours, the 328 $m\mu$ band had largely disappeared and had been replaced by new absorption in the region 290 $m\mu$. At this wave-length the intensity remained practically constant at $E_{1\text{cm.}}^{1\%}$ = 370 for a further 54 hours' heating, when the experiment was stopped; the definite maximum at 290 $m\mu$ attaining its highest persistence (never very great) after about 10 hours. The minimum was also fairly constant at $E_{1\text{cm.}}^{1\%}$ 265 $m\mu$, 330. The narrow absorption bands first became detectable after about 10 hours, and reached the highest definition a few hours later, but at best occurred with far less clarity than in the experiments with alcoholic hydrogen chloride or in the fractional slow high vacuum distillation, being little more than fairly sharp inflexions. Substantially, then, the prolonged heating at 120–125° brings about a type of

decomposition associated much more with a substance exhibiting the 290 $m\mu$ maximum than with the narrow bands. If, therefore, the occurrence of these bands in certain distillates is due to heat alone, temperatures above 125° are seemingly essential, and possibly the reaction occurs in the vapour phase. It is interesting to note that while the distillates consisted of pale oils, in our experiments the material grew more and more viscous as the heating progressed, eventually becoming a clear red resin softening at 40–50°. Determinations of the colour test intensities (antimony trichloride) indicated a steady decrease in vitamin A content, but even after 60 hours a perceptible blue colour was given although the intensity had fallen to about 1/40 of the initial value.

Oxidation of vitamin A.

There is general agreement in the literature of the subject that oxygen should be excluded as completely as possible in order to minimise loss during the processes of saponification and extraction of the vitamin from liver oils. During recent months we have repeatedly observed, however, that rich concentrates exhibit a somewhat unexpected resistance to oxidation under certain conditions, and Dann [1931; 1932] has already shown that oxidation of vitamin A concentrates by aeration at the boiling-point in alcohol or alcoholic potash is not achieved in 24 hours. Our own experiments have been carried out on rich halibut-liver oil concentrates dissolved in alcohol. A moderately brisk stream of oxygen from a cylinder was passed through the solution heated under reflux and samples were withdrawn periodically, the vitamin A potency being determined in terms of the intensity of the ultra-violet absorption maximum at 328 $m\mu$. For 8–10 hours the absorption spectrum remained quite constant within experimental error (here $\pm 5\%$), but after this period the intensity of absorption at 328 $m\mu$ slowly decreased and fine absorption bands with maxima near 392, 369 and 350 $m\mu$ began to appear, not, however, with any great intensity. During the experiment there is a very decided increase in the intensity of absorption on the ultra-violet side of 328 $m\mu$ and this is reflected in a large decrease in persistence. The simplest interpretation of the results is that after a prolonged period of induction slow oxidation occurs coinciding with two changes in spectral absorption, one the appearance of rather unselective absorption in the middle ultra-violet and the other the appearance of highly selective absorption on the long wave side of 328 $m\mu$. The experiments confirm Dann's observation of the high resistance to oxidation of alcoholic solutions of vitamin A, and indicate that the first oxidation product probably exhibits a broad unresolved band near 290 $m\mu$.

As would be expected, ozone is much more effective than oxygen and the vitamin is readily destroyed. In our experiments the object was to ascertain whether any new absorption bands could be recorded after the vitamin had been exposed for a few seconds to small quantities of ozonised oxygen. The reaction proved very difficult to control but certain qualitative observations are of interest. In the first place, exposure of dilute alcoholic solutions of

vitamin A to ozonised oxygen invariably results in a substantial decrease in the intensity of absorption at $328\text{ m}\mu$, but the intensity in the region 250 to $300\text{ m}\mu$ does not fall nearly so rapidly, so that the reaction products must absorb more strongly in the middle ultra-violet than in the region $300\text{--}400\text{ m}\mu$. In a number of cases, the exposure to ozonised oxygen was of very short duration, and it seems that the first spectroscopically detectable oxidation (ozonisation) product is characterised by a broad absorption band of small persistence, free from "fine" structure, and exhibiting a maximum at $290\text{ m}\mu$ approx. The intensity of this absorption band is of the same order as that of vitamin A at $328\text{ m}\mu$. In other experiments a maximum at $272\text{ m}\mu$ made its appearance. In all experiments in which a solution of rich concentrate (0.008% , $E_{1\text{cm}}^{1\%}$ $328\text{ m}\mu$, 1100) was exposed to a fairly slow stream of ozonised oxygen for more than 1 minute at room temperature the resulting solution showed no appreciable absorption over the range $200\text{--}500\text{ m}\mu$. In no circumstances was it possible to observe narrow, sharply defined absorption bands as a result of the action of ozone on vitamin A concentrates.

A complete interpretation of the foregoing results is impossible at this stage, but it may perhaps be useful to point out a distinctly interesting analogy emerging from this work and that of Kuhn [1931] on unsaturated compounds [cf. also Euler *et al.*, 1932]. Crotonic acid has a broad band with a maximum at $204\text{ m}\mu$, sorbic acid has a similar band with its maximum displaced to $250\text{ m}\mu$, whilst the octatriene and decatetraene acids exhibit bands at $294\text{ m}\mu$ and $329\text{ m}\mu$ respectively (private communication from Prof. Kuhn). Similarly Euler *et al.* [1932] record continuous end absorption on the ultra-violet side of $250\text{ m}\mu$ for sorbic alcohol ($\text{CH}_3(\text{CH}=\text{CH})_2\text{CH}_2\text{OH}$), a well defined maximum near $270\text{ m}\mu$ ($\log \epsilon 4.75$) for octatrienol ($\text{CH}_3(\text{CH}=\text{CH})_3\text{CH}_2\text{OH}$) and a maximum near $310\text{ m}\mu$ ($\log \epsilon 4.7$) for decatetraenol ($\text{CH}_3(\text{CH}=\text{CH})_4\text{CH}_2\text{OH}$). Now, it seems significant that the first oxidation product of vitamin A exhibits a maximum near $290\text{ m}\mu$ and the second product a maximum near $272\text{ m}\mu$. By analogy with the data quoted above it is likely that the selective absorption of vitamin A is due largely to a straight chain containing four conjugated double bonds and that the displacement from 310 (decatetraenol) to $328\text{ m}\mu$

Table III.

Material	λ max. ($\text{m}\mu$)	*log ϵ max.
Crotonic acid	204	4.08
Sorbic acid	250	4.40
Octatriene acid	294	4.56
Decatetraene acid	329	4.69
Sorbic alcohol	< 240	order 5
Octatrienol	270	4.75
Decatetraenol	310	4.7
Vitamin A	328	4.5 circa
		(calculated on a molecular weight near 300)
1st oxidation product	290	order 4.5
2nd oxidation product	272	—

* ϵ is molecular extinction coefficient.

(vitamin A) is due to the joint action of the fifth double bond and the "loading" effect of the ionone ring. Probably, the first action of ozone is to attack one of the double bonds in the side chain, and then selectively to attack a second. It is interesting that only two intermediate stages in the oxidation can be detected spectroscopically. This is not surprising since sorbic acid has its maximum well in the ultra-violet and sorbic alcohol probably in the more extreme ultra-violet.

Table III illustrates the main points referred to above.

DISCUSSION AND SUMMARY.

The recovery of vitamin A from the blue solution obtained with antimony trichloride by pouring the mixture into much water has been attempted. Partial decomposition of the vitamin occurs, accompanied always by the appearance of narrow absorption bands with maxima at 399, 376, 357 and $340 m\mu$ (chloroform), and sometimes by additional, less definite maxima, *e.g.* at 425, 324, 308 and $280 m\mu$. The wave-lengths at the maxima are constant, irrespective of whether either rich or poor oils, or concentrates are used, but the intensities vary roughly with the potency of the material. Recovery of vitamin A, although never quantitative, supports the view that the initial reaction is the formation of a vitamin A-antimony trichloride loose addition product, but secondary (condensation?) processes cannot be excluded, and probably account for the narrow bands. This view is supported by the observation that the similar maxima 392, 369, 350, $333 m\mu$ (alcohol) can be obtained with greatly enhanced definition by the action of alcoholic hydrogen chloride on rich concentrates, the intensity, persistence, rate of development and permanence depending on the concentration of hydrogen chloride. When the narrow bands are best shown, the $369 m\mu$ band is the most intense but even under optimum conditions (15° , $N/30$ HCl, 20 minutes) the reaction is incomplete and the product unstable in acid. Neutralisation of the acid with sodium ethoxide arrests the decomposition of the material, but does not effect reversal to vitamin A.

Similar narrow bands, together with a broad continuous band with its maximum at $290 m\mu$ are shown in certain fractions obtained in slow high vacuum distillation of rich concentrates heated to about 180° . By prolonged heating at 120 – 125° in an atmosphere of nitrogen the development of narrow bands could not be induced to more than a minute extent, but the vitamin nevertheless decomposed fairly readily, the main product exhibiting a maximum at $290 m\mu$. At the boiling-point in alcohol, vitamin A is unexpectedly stable to prolonged exposure to a stream of oxygen. After some hours, the $328 m\mu$ maximum decreases in intensity, the absorption in the region 260 – $300 m\mu$ slowly increases and the narrow absorption bands in the near ultra-violet appear, but with comparatively low persistence. Ozonised oxygen rapidly destroys vitamin A with disappearance of selective absorption but compounds with maxima at $290 m\mu$ and $272 m\mu$ can be detected in the very early stages.

The data are consistent with the vitamin A molecule containing a side chain with at least four double bonds, which can be attacked in turn by ozone to give products with broad bands.

The hypothesis of ring closure to give hydronaphthalene derivatives as an explanation of the narrow bands will be discussed in a later communication.

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