XXIV. STUDIES IN THE STABILITY ON VITAMINS A AND D

II. ACTION ON FATTY PEROXIDES ON VITAMIN A

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THE present widespread use of vitamin A in medicine calls for information as to its stability, particularly when dissolved in oils. Several workers [Huston *et al.* 1928; Evers, 1929; Norris & Church, 1930; Holmes *et al.* 1936] have studied the stability under different conditions of storage of vitamin A in fish liver oils and of vitamin A concentrates dissolved in vegetable oils. Wide variations in the rate of destruction of vitamin A have been recorded: Jones [1928] found a reasonable amount of vitamin A present in a malt extract and cod liver oil preparation 23 years old; at the other extreme Marcus [1931] found vitamin A to be largely destroyed in 3 hr. when dispersed on decolorizing charcoal. Other workers [Wokes & Willimott, 1927; MacWalter, 1934] have studied the rate of destruction of vitamin A in cod liver oil aerated at various temperatures. Dann [1932] investigated the rate of destruction of vitamin A in a cod liver oil concentrate dissolved in a range of solvents, on aeration at 98°, and found surprising variations from complete stability in certain alcohols and esters to rapid destruction in fatty acids. Fridericia [1924] and Powick [1925] found that admixture with rancid lard slowly destroyed vitamin A in cod liver oil, and suggested that the effect might be due to organic peroxides. Rosenheim & Webster [1926] found peroxides present in cod liver oil in which the vitamin A had disappeared on exposure to air and light. Wokes & Willimott [1927] suggested that the destruction of vitamin A caused by aerating cod liver oil was due to the formation of volatile organic peroxides, while Whipple [1936] attempted to correlate the peroxide value with the destruction of vitamin A in cod liver oil as rancidity developed. On the other hand, Griffiths *et al.* [1933] found that vitamin A was stable for at least 4 months in cod liver oil emulsions prepared with gum acacia containing peroxidase. Cady & Luck [1930] found that emulsification of cod liver oil with H₂O₂ only slowly destroyed the vitamin A present, and Dann [1932] found that vitamin A was stable in a cold alcoholic solution of H₂O₂ for 24 hr.

We have made a number of experiments on the stability of vitamin A (in the form of concentrates) dissolved at various strengths in various vegetable oils, with and without the addition of 0.05% quinol as antioxidant. In all instances the vitamin A content (measured by the absorption at 328 mμ) diminished, sometimes to vanishing point, during the course of 2 years' storage, but the rates of destruction were extremely erratic, even to the extent that the same solution in different bottles deteriorated at different rates. In general, there was a tendency for the more dilute solutions (1200 and 12,000 I.U./g.) to deteriorate more rapidly than the stronger ones (60,000 I.U./g.). In some instances quinol

had a slight retarding effect on the destruction but in others it appeared inactive. This is contrary to the findings of Norris & Church [1930] and of various other workers who have used quinol to protect the vitamin A in solutions for animal feeding, but it must be noted that the solutions we tested were very much more concentrated. Many of the solutions were rancid at the end of the test, but vitamin A was still present in some of these rancid solutions, whereas little or none was present in some of the solutions which neither tasted nor smelt rancid. Nevertheless, it seemed that the most probable explanation of the erratic nature of these findings was that fatty peroxides were the agents immediately destructive of the vitamin A, since it is well known that the rate of development of peroxides in oils is affected by conditions of storage, the extent of exposure to air, and particularly by the amount of natural antioxidants present.

A number of samples of various vitamin A concentrates at a uniform strength of 12,000 I.U./g. in arachis oil stored for many months in partly filled bottles were available. These were examined both for the content of vitamin A and of peroxides (by a slight modification of Lea's method [1929]). It was found that always when the peroxide value exceeded 5, at least 70 % of the vitamin A had disappeared. All samples in which little destruction had occurred showed very low peroxide values, but nevertheless there were several samples with low peroxide values in which considerable destruction of vitamin A had occurred. It is, of course, possible that these samples had at some time contained more peroxides, which had been used up in destroying the vitamin A, or in oxidizing constituents of the oil.

A number of experiments were then made in order to test more rigorously the theory that fatty peroxides slowly oxidize vitamin A. In the first series, a number of samples of vitamin A in the form of rich fish liver oils and concentrates of different types were dissolved to a strength of 12,000 I.U./g. in a rancid olive oil having a peroxide value of 260.¹ The solutions were immediately transferred to a series of glass tubes, which were evacuated, filled with N₂ and sealed. The tubes were then heated in a boiling water bath, and at intervals a tube was removed and the vitamin A determined by measuring the absorption at 328 m μ . It was observed that a progressive bleaching of the solutions occurred, while the absorption at 328 m μ steadily diminished with time of heating. Further, the trough of the absorption curve on the short-wave side of 328 m μ became filled up as the reaction proceeded, until the later samples showed only general absorption. In every instance the actual absorption at 328 m μ was measured, irrespective of whether or not the curve showed a maximum at this wave-length. All values were corrected for the slight general absorption of the solvent oil at 328 m μ . Every experiment was done at least in duplicate. The results are shown graphically in Fig. 1. It will be observed that the curves fall into 2 groups. The curves corresponding with the more rapid destruction of vitamin A cover 3 samples of vitamin A concentrate, 2 from fish liver oil and 1 from mammalian liver. The curves corresponding with slower destruction of vitamin A cover two fish liver oils (one of them of exceptionally high potency). It has previously been shown [Bacharach & Smith, 1928] that vitamin A exists in fish liver oil in a state different from that in which it is present in concentrates, and it was suggested that vitamin A is an alcohol, which in fish liver oils is esterified with fatty acids. It is interesting to note that this difference is reflected in a greater resistance of the esterified vitamin A to oxidation by fatty peroxides. The matter was further tested by esterifying a vitamin A concentrate with stearic acid, and

¹ As defined by Lea; each g. of oil contained peroxide oxygen equivalent to 260 ml. of N/500 thiosulphate.

measuring the rate of destruction of the vitamin A by the rancid olive oil. The data are included in Fig. 1, and it will be seen that the points lie close to those for the fish liver oils.

Fig. 1.

Fig. 1 shows clearly that the absorption at 328 $m\mu$ tends not towards zero, but towards a value of some 10–20 % of the initial absorption. The probable explanation of this phenomenon is that the primary reaction concerns only one of the double bonds in the vitamin A molecule, and that the primary oxidation product has an absorption band at a shorter wave-length than that due to vitamin A, but broad enough to show significant absorption at 328 $m\mu$. Some confirmation of this was afforded by the $SbCl_3$ colour test. A solution in rancid oil after 8½ hr. heating showed 11 % of the initial absorption at 328 $m\mu$; the colour test indicated only 7 % of the initial vitamin A and, in addition, the test was abnormal; the solution became purple on adding the reagent, and the colour rapidly changed to blue, the reverse of the normal change. It is quite conceivable that some such colour reaction would be given by a primary oxidation product of vitamin A. There is some indication from the shape of the curves, in particular that for the artificially esterified vitamin A, that this primary oxidation product is itself slowly attacked by peroxide oxygen, with the production of a substance showing less absorption at 328 $m\mu$. It is certainly possible for vitamin A to be oxidized to a substance almost completely transparent at 328 $m\mu$, for we have had oil solutions decrease in absorption with development of rancidity to 1.5 % of the initial value, and this residual absorption was probably due to the glycerides.

In order to prove more definitely that the fatty peroxides themselves and not some other constituent of rancid oil were the actual destructive agents, a series of oils were prepared having different peroxide values. Olive oil was chosen as a typical vegetable oil and coconut olein as an oil mainly containing shorter carbon chains. Samples of these oils were exposed to the air in a warm place, while others were heated at 100° and others irradiated with a quartz

mercury vapour lamp. A sample of vitamin A concentrate was dissolved in each of the oils to a strength of 9600 I.U./g. and the samples were sealed in N_2 as before. They were all heated for the same times, namely, 2 and $4\frac{1}{2}$ hr. at 100° and 45 hr. at 50° . The final vitamin A contents are shown in Table I, where the

Table I. *Fish liver oil concentrate dissolved in oils to 9600 I.U./g.*

Oil	Peroxide value ml. $N/500$ $Na_2S_2O_3$ per g.	% original $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ after		
		2 hr. at 100°	$4\frac{1}{2}$ hr. at 100°	45 hr. at 50°
Coconut olein V	0.5	96	—	95
Coconut olein VI	4.8	90	82	(87)
Olive oil I	10.5	83	81	94
Coconut olein VII	15	84	59	—
Olive oil II	18	79	65	96
Coconut olein VIII	36	74	57	87
Coconut olein IX	50	57	45	73
Olive oil III	64	57	39	73
Olive oil IV	125	41	22	63
Undiluted concentrate		94	92	96

oils are arranged in order of increasing peroxide value, irrespective of their nature (olive oil or coconut olein) or the method by which peroxide formation was induced. It will be observed that with very few exceptions this order is also the order of decreasing vitamin A content in the heated samples. The experiment was repeated with a sample of artificially esterified concentrate dissolved in some of these oils at a strength of 12,000 I.U./g. (Table II). No measurable destruction

Table II. *Esterified fish liver oil concentrate dissolved in oils to 12,000 I.U./g.*

Oil	Peroxide value ml. $N/500$ $Na_2S_2O_3$ per g.	% original $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ after		
		2 hr. at 100°	$4\frac{1}{2}$ hr. at 100°	100 hr. at 45°
Coconut olein V	0.5	100	100	—
Coconut olein VI	4.8	—	100	98
Olive oil I	10.5	100	100	100
Coconut olein VIII	36	91	88	88
Olive oil III	64	83	79	97
Olive oil IV	125	75	70	84

occurred, except with oils having a peroxide value of 36 or higher, but again (with one exception) increasing peroxide value went parallel with decreasing vitamin A; destruction was again much slower than with the unesterified concentrate.

No attempt was made to confirm these findings by biological assays, mainly because it was to be expected that the fatty peroxides would cause further destruction of vitamin A during the course of the assay or during any attempt to remove the peroxides, e.g. by saponification.

Some preliminary experiments along similar lines were done with solutions of calciferol in rancid olive oil, but it was impossible to assay the vitamin D accurately. Biological assays were contra-indicated, for the reason mentioned in connexion with vitamin A. There were indications that the oxidation products of vitamin D interfere with its direct spectrophotometric determination and even more with its determination by the Brockmann method with $SbCl_3$. Nevertheless, these experiments showed that calciferol is far more resistant than is vitamin A to destruction by fatty peroxides. Conditions that caused almost complete destruction of vitamin A caused barely 10% destruction of vitamin D.

DISCUSSION

In most of these experiments the concentration of peroxide oxygen was vastly in excess of that of the vitamin A, so that the reaction would be expected to follow a unimolecular, i.e. logarithmic law. When appropriate allowances were made for the absorption at $328\text{ m}\mu$ of the first oxidation product postulated, the experimental results were found to follow approximately the logarithmic law.

In the second series it would be expected that the velocity of reaction would be proportional to the peroxide value. This was found to be the case within the limits of error of the spectroscopic determinations, except that the vitamin A in the solutions of low peroxide value was destroyed more rapidly than was to be expected. The indication here is that some factor other than peroxide oxygen caused very slow destruction of vitamin A. The fact that slow destruction of the undiluted vitamin A concentrate occurred when it was heated in N_2 -filled tubes alongside the oil solutions suggests that this factor is simple heat. Edisbury *et al.* [1932] and Robinson [1938] have shown that heat can cause destruction of vitamin A in absence of O_2 or any oxidizing agent.

CONCLUSIONS

These experiments appear to afford clear evidence that fatty peroxides can cause destruction of vitamin A rapidly at 100° and more slowly at 50° and at room temperature. A practical consideration which follows is that the peroxide value of oil solutions of vitamin A needs to be considered, not merely as an index of potential rancidity, but as an index of the stability of the vitamin A. Moreover, a peroxide value not high enough to cause concern as to the stability of the oil itself may well be sufficient to cause slight destruction of dissolved vitamin A. The evidence that is here presented confirms that given in the previous paper of this series [Robinson, 1938] to the effect that oxidation products of vitamin A may show considerable absorption at $328\text{ m}\mu$. It follows that with vitamin A preparations that have suffered oxidation, a considerable portion of the absorption at $328\text{ m}\mu$ may be due to substances other than vitamin A. Thus the present work affords further evidence that a spectrophotometric assay must be regarded with some suspicion when the band at $328\text{ m}\mu$ shows poor persistence.

Fatty acid esters of vitamin A are more stable than the free alcohol towards fatty peroxides. This might be due either to the participation in the reaction of the hydroxyl group present in the free alcohol or, perhaps more likely, to activation by the hydroxyl group of the conjugated double bond system, rendering it more susceptible to attack by peroxide oxygen.

SUMMARY

Vitamin A held in oil solution at room temperature disappeared at widely varying rates, but in general, destruction was greatest in solutions that developed a high peroxide value. Liver oils and concentrates were dissolved in rancid olive oil of peroxide value 260 and heated to 100° in N_2 -filled sealed tubes. Progressive destruction of vitamin A occurred, more rapidly with the concentrates (of fish and mammalian origin) than with the fish liver oils (in which vitamin A occurs as an ester) or a re-esterified concentrate.

Oxidation of the vitamin A gave rise to a product with unselective absorption at $328\text{ m}\mu$, so that when oxidation has occurred the total absorption at $328\text{ m}\mu$ indicates values in excess of the true vitamin A content.

Two concentrates were dissolved in samples of olive oil and coconut olein showing a wide range of peroxide values and the solutions sealed into tubes. After heating of the tubes under identical conditions, the extent to which the vitamin A had been destroyed was approximately proportional to the peroxide value and independent of the nature of the oil.

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