CIX. STUDIES IN THE STABILITY OF VITAMINS A AND D. I

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ALTHOUGH it is well known that vitamin A is destroyed by the action of air and heat, little information is available about the changes that occur during this destruction. The joint effect on the vitamin of heat and air or oxygen has accordingly been investigated.

Measurement of O₂ absorption

The uptake of O₂ by the unsaponifiable fraction (I) of a vitamin A-rich oil was measured by a method similar to that used for linseed oil by Rhodes & van Wirt [1923] and for butter fat by Holm & Greenbank [1923]. An accurately weighed amount of the concentrate, which contained about 50 % of vitamin A (estimated spectrophotometrically), was spread on strips of filter paper, which were dropped into a flask. This was then connected to a mercury manometer, the entire apparatus was filled with O₂ and the amount of O₂ absorbed when the flask was heated at 100° was noted at intervals. Absorption was at first rapid, but slowed down appreciably after about 3 hr. The experiment was terminated at the end of 5½ hr., during which time nearly 50 ml. or 0.06 g. O₂ had been absorbed per g. concentrate. The oxidized material was extracted from the filter paper with ethyl alcohol and the solution was examined spectrophotometrically. It was found that the absorption band no longer had a maximum at 328mμ and that the value of $E_{1\text{ cm.}}^{1\%}$ 328mμ had fallen from 700 to 100. The destruction of vitamin A by heat in the absence of O₂ was shown to be a relatively slow process, since when the same concentrate was heated in a high vacuum for 4 hr. at 120°, the value of $E_{1\text{ cm.}}^{1\%}$ 328mμ fell to 530 only.

That heat plays only a secondary part in the reaction was confirmed by a different type of experiment, in which a small amount of concentrate I was mixed with 2.5 times its weight of bile salts and heated in O₂ as before. The uptake of O₂ was negligible, and the vitamin A appeared to have been protected completely from oxidation, presumably by the deoxycholic acid present in bile salts. The vitamin A was recovered and examined spectrophotometrically, when it was found that the value of $E_{1\text{ cm.}}^{1\%}$ 328mμ had only fallen from 700 to 610 after 4 hr. at 100°. This reduction is of the same order as that brought about by heating the concentrate *in vacuo* at this temperature, so that the bile salts had protected the vitamin from inactivation by oxidation but not by heat. The behaviour recalls the protection from autoxidation of benzaldehyde in combination as a choleic acid. Shimizu & Hatakeyama [1929] prepared a crystalline compound of vitamin A and deoxycholic acid, which they called gallosterol; they reported that it was stable in air.

Physical, chemical and biological properties of vitamin A oxidation products

An attempt to determine the mechanism of the oxidation of the vitamin A concentrate was made by carrying out analyses on a further quantity of the oxidized material. Iodine values were determined both with Wijs's reagent and with the pyridine-sulphate-dibromide reagent of Rosenmund & Kuhnenn [1923]. Identical results were obtained. Acetyl values (mg. CH_3CO required to combine with the alcohols in 1 g.) were determined with acetic anhydride-pyridine mixture by the method of Verley & Bölsing [1901]. Peroxide values (ml. $N/500 \text{ Na}_2\text{S}_2\text{O}_3$ per g.) were determined by a method similar to that of Lea [1931], in which approximately 0.3 g. of the substance was dissolved in 10 ml. chloroform and 10 ml. acetic acid, the solution was boiled for 1 min. with a mixture of 0.5 ml. 50 % KI solution, diluted with water and then titrated with $N/500 \text{ Na}_2\text{S}_2\text{O}_3$. It has since been found that when used with a highly unsaturated substance such as vitamin A this method gives low results, because some of the liberated iodine is absorbed by the vitamin. The true peroxide values of these substances are therefore higher than those recorded in the present communication.

It was found that after heating another portion of concentrate I at 100° in air for 5 hr., the value of $E_{1\text{ cm.}}^{1\%}$ $328\text{ m}\mu$ had fallen from 700 to 200, the iodine value from 231 to 176 and the acetyl value from 130 to 96 mg. acetyl per g., but the peroxide value was negligible. The presence of a small amount of aldehyde or ketone was indicated by a loss in weight when the material was treated with *o*-carboxyphenylhydrazine followed by extraction of the ethereal solution with alkali. Ultimate analyses indicated that each g. of concentrate had combined with 0.02 g. O_2 , which is much less than the amount of O_2 absorbed in the previous experiment. The biological activity was only 100,000 I.U./g., whereas from the absorption spectrum the activity should have been 320,000 (using the factor of 1600 for converting from values of $E_{1\text{ cm.}}^{1\%}$ $328\text{ m}\mu$ to I.U.). Thus the conversion factor for this material is only 500. With a highly oxidized vitamin A concentrate, therefore, the spectroscopic assay gives values greatly in excess of those obtained by biological assay.

These results were sufficiently interesting to prompt further and more exhaustive work with a richer concentrate (II), having a value of 1070 for $E_{1\text{ cm.}}^{1\%}$ $328\text{ m}\mu$, equivalent (using the conversion factor of 1600) to nearly 70 % vitamin A. This was oxidized in stages, thin films of the material being heated in air at 100° for 3, 9, 24 and 48 hr. The changes that occurred in the absorption spectrum of the concentrate on progressive oxidation are shown in Fig. 1. It will be observed that not only was the maximum of the band progressively depressed, but it was displaced from $328\text{ m}\mu$ to about $310\text{ m}\mu$ in the material heated for 9 hr., and to about $290\text{ m}\mu$ in the material heated for 24 hr. In the material heated for 48 hr., there was hardly any sign of the original band.

Not only were ultimate analyses and determinations of the iodine value, acetyl value and peroxide value carried out on the original concentrates and on each of the heated concentrates, but Carr-Price values, acid and saponification values and molecular weights were also determined. An attempt was also made to ascertain the ketone and aldehyde contents by the use of reagent P of Girard & Sandeleseu [1936]. The results of all these estimations are given in Table I and they confirm those obtained in the previous experiment. The iodine values, indicating the degree of unsaturation, became progressively smaller, as did the acetyl values. The acid values remained constant, but, rather unexpectedly, the saponification value increased. The small initial peroxide value

disappeared as oxidation proceeded, although the same concentrate exposed to air at room temperature acquired a high peroxide value in a relatively short time. The possibility of the formation of peroxides which immediately decompose on heating cannot be excluded. The amounts of aldehydes and ketones increased to quite large proportions, but it was not possible to isolate crystalline derivatives. The percentage of total O_2 showed a regular increase. The distribution of the O_2 in the four lots of material is given in Table II. It should be

Fig. 1. Absorption curves of vitamin A concentrate and its four oxidation products. I, original concentrate; II, concentrate heated for 3 hr.; III, concentrate heated for 9 hr.; IV, concentrate heated for 24 hr.; V, concentrate heated for 48 hr.

Table I. *Physical, chemical and biological properties of vitamin A concentrate and its oxidation products*

	Original concentrate	Oxidation product			
		1 (3 hr.)	2 (9 hr.)	3 (24 hr.)	4 (48 hr.)
Biological activity (I.U./g.)	1130×10^3	770×10^3	290×10^3	50×10^3	—
$E_{1\text{ cm}}^{1\%}$ (328mμ)	1070	810	450	270	62.5
Spectroscopic assay (I.U./g.)	1700×10^3	1300×10^3	720×10^3	430×10^3	68×10^3
Carr-Price value	56,000	34,000	14,000	5000	370
Colour test assay (I.U./g.)	1740×10^3	1050×10^3	430×10^3	150×10^3	10×10^3
Iodine value	284	267	251	195	—
Acetyl value	122	118	106	103	—
Acid value	8	8	8	7	—
Saponification value	12	17	33	41	—
Peroxide value	9	5	1	1	—
Ketones %	15	25	28	45	—
Non-carbonyl compounds %	85	68	60	20	—
Aldehyde % (by diff.)	0	7	12	35	—
Mol. wt. benzene c. 1 %	350	—	410	780	—
c. 3 %	380	—	500	860	—
Mol. wt. acetic acid c. 1 %	280	—	355	200	—
c. 3 %	260	—	355	300	—
Carbon %	83.1	83.0	81.1	79.7	—
Hydrogen %	10.7	10.7	10.3	9.9	—
Oxygen %	6.2	6.3	8.6	10.4	—

Table II. *Distribution of oxygen in vitamin A concentrate and its oxidation products*

	Total O ₂ % (from ultimate analyses)	Alcohol O ₂ % (from acetyl values)	Acid O ₂ % (from acid values)	Ester O ₂ % (from saponifi- cation values)	Ketone + aldehyde + ether O ₂ % (by difference)
Original concentrate	6.2	4.55	0.45	0.65	0.55
Concentrate heated for 3 hr.	6.3	4.4	0.45	0.95	0.5
Concentrate heated for 9 hr.	8.6	3.95	0.45	1.9	2.3
Concentrate heated for 24 hr.	10.4	3.85	0.4	2.35	3.8

noted that, assuming there has been no loss of carbon, the material after 24 hr. heating has gained 5.5 parts of O₂ per 100 parts of C and lost 0.5 part of H₂, presumably as water. This, however, would require 4.0 parts of O₂, so that actually 9.5 parts of O₂ were involved in the oxidation of the vitamin concentrate. This explains the discrepancy previously noted between the amount of O₂ absorbed and that found to be in combination.

The original concentrate II was a slightly viscous orange-red liquid, the first oxidation product was a highly viscous oil, the other oxidation products were hard resins resembling shellac. That polymerization had occurred was confirmed by measurements of the "molecular weights" of three of the materials. With benzene as solvent the actual values varied slightly according to the concentration used, but the most highly oxidized material had the highest "molecular weight". With acetic acid as solvent irregular results were obtained. Similar irregularities were observed with oxidized linseed oil by Gay [1933], who attributed them to interaction between the solvent and solute. The figures obtained by the use of benzene can be regarded as giving an approximate indication of the average weight of the polymerized aggregates, though the true values are probably higher.

DISCUSSION

The analytical data obtained indicate that the original concentrate II contained 80% of alcohol calculated as C₂₀H₃₀O, together with appreciable amounts of acid, ester, and ketone or aldehyde. From the biological activity of the material, however, it is evident that by no means all the alcohol present was vitamin A (C₂₀H₃₀O), so that a considerable amount of impurity must have been present. For this reason, the mechanism here proposed for the oxidation of vitamin A must be accepted with some caution, and can only be verified when a quantity of pure vitamin A is available for investigation.

The main changes occurring during the oxidation at 100° of the vitamin A concentrate were loss of hydrogen, decrease in hydroxyl, marked increase in the amounts of ester, aldehyde and ketone, increase in the "molecular weight" and, finally, an increasing degree of saturation.

It is quite clear, therefore, that the hydroxyl group of vitamin A cannot be inert, but is oxidized to aldehyde; this would involve a loss of hydrogen. The presence of "vitamin A aldehyde" itself, however, is precluded, since this would be expected to give rise to absorption bands at about 340 mμ. Thus a breakdown of the molecule must occur either simultaneously with or prior to oxidation. Esters might be formed by such aldehydes condensing together. The possibility cannot be excluded, however, that the saponification value of the oxidized concentrate is due, not to the presence of ester, but to a Cannizzaro reaction brought about by the actual process of "saponification"; both purified benzaldehyde and crotonaldehyde possess "saponification values".

Ellis [1926; 1936] concluded that double bonds are oxidized in one of two ways, either to peroxides by way of epi-oxides, or to $\alpha\beta$ -unsaturated glycols and ketohydroxy compounds, these being tautomeric. The second possibility seems unlikely in the oxidation of vitamin A, since a decrease in the amount of hydroxyl was observed as oxidation progressed, instead of an increase as was found for the substances investigated by Ellis [1926] and by Hamilton & Olcott [1937]. The double bonds of vitamin A, therefore, are probably oxidized to peroxide groups, which at 100° break down as quickly as they are formed. Thus by reaction with unsaturated groups they would give rise to polymerides [Morrell & Davis, 1936], and by disruption they would result in the formation of aldehydes or ketones. It is probable that both types of reaction occur during the oxidation of vitamin A. The first reaction would lead to an increase in the "molecular weight", and the second reaction would lead to the accumulation of aldehydes and ketones in the highly oxidized material.

The changes that have been found to occur during the oxidation of a vitamin A concentrate by atmospheric oxygen at 100° can therefore best be explained on the assumption that the following reactions occur more or less simultaneously:

(1) Addition of O₂ to any or all of the double bonds with formation of peroxides, which are immediately converted into other substances.

(2) Disruption of the peroxides giving aldehydes and ketones (and possibly also cyclic ethers and O₂).

(3) Reaction of the peroxides with unsaturated linkages giving polymerized substances.

(4) Oxidation of the alcohol group giving an aldehyde.

Such a mechanism would explain the decrease in hydrogen content, iodine value and acetyl value, and the increase in aldehyde and ketone content and molecular weight. It is consistent with the failure to find appreciable amounts of peroxide, which is presumed to be destroyed as quickly as it is formed. It does not, however, explain the increase in the saponification value, which is at present held to be due to an increase in the amount of aldehyde rather than of ester.

Biological changes resulting from oxidation

Bio-assays were carried out on concentrate II and on its oxidation products 1, 2 and 3. The test solutions were assayed spectrophotometrically before and after feeding and only one of the solutions, that of oxidation product 1, showed deterioration during the test, and this amounted to only about 10%. The results of the animal assays are given in Table III. It will be seen from Tables I

Table III. *Biological activity of vitamin A concentrate and its oxidation products*

	Biological activity (I.U./g.)	No. of animals used	$E_{1\text{ cm.}}^{1\%}$ 328m μ	Conversion factor
Concentrate I heated for 5 hr.	130,000	16	203	650
Concentrate II unheated	1,130,000	10	1070	1060
		(Males only)		
Concentrate II heated for 3 hr.	770,000	38	810	950
Concentrate II heated for 9 hr.	290,000	18	450	650
Concentrate II heated for 24 hr.	50,000	10	270	190

and III and from Fig. 1 that the biological activity, the value of $E_{1\text{ cm.}}^{1\%}$ 328m μ and the Carr-Price value decrease progressively and that the relation between

them is not constant. The factors for conversion of $E_{1\text{ cm.}}^{1\%}$ 328m μ into international units found for the concentrate and its first three oxidation products were respectively 1060, 950, 650, 190; the factors for conversion of Carr-Price values into international units were 21.5, 22.5, 20.5, 10 respectively. Thus by making use of the accepted factors of 1600 and 31, unjustifiably high estimates of vitamin A contents would be obtained. The ratios Carr-Price value/ $E_{1\text{ cm.}}^{1\%}$ 328m μ were found to be 52, 42, 31, 18 and 5 for the original concentrate and its four oxidation products respectively.

Although the approximate accuracy of the 1600 factor has recently been confirmed for a halibut liver oil and its non-saponifiable fraction carefully prepared under laboratory conditions [Hume, 1937], a number of workers have found lower and variable values for the factor, particularly for commercial concentrates [cf. Bacharach *et al.* 1936]. Table IV shows some of the values

Table IV. *Biological activity of vitamin A concentrates*

Type of concentrate	Biological activity (i.u./g.)	No. of animals used	$E_{1\text{ cm.}}^{1\%}$ 328m μ	Conversion factor
1 Fish	400,000	26	380	1060
2 Fish .	1,200,000	30	975	1230
3 Fish	460,000	18	460	1000
4 Fish	560,000	14	620	920
5 Fish	570,000	18	520	1100
6 Fish and mammalian	270,000	16	306	875
7 Fish and mammalian	300,000	16	351	860
8 Fish and mammalian	390,000	16	392	1000
9 Fish	350,000	20	422	840
10 Fish and mammalian	395,000	16	341	1160
11 Fish	740,000	14	585	1260
12 Fish	570,000	24	555	1024
13 Fish	360,000	30	433	830
14 Fish	540,000	22	558	970

obtained in our own laboratories for commercial concentrates. The values obtained by different workers for the three ratios biological assay/ $E_{1\text{ cm.}}^{1\%}$ 328m μ , biological assay/Carr-Price value and Carr-Price value/ $E_{1\text{ cm.}}^{1\%}$ 328m μ have been calculated from their published results and are shown in Table V. With the exception of four oils, which include oils "X" and "Z", there is no instance published in the literature of any material for which the first ratio exceeds 1600. On the other hand, most of the oils give values of 1000 to 1400 and mammalian liver oils give even lower values; the concentrates invariably give lower values. The factor for converting Carr-Price units into international units is also significantly lower for concentrates than for oils. Unfortunately, no information is given in the literature about the previous history of these oils and concentrates, and it is possible that many of the oils had already attained high peroxide values and that the concentrates, especially where described as "commercial", had been exposed to heat and air. We believe that both stale fish liver oils and commercial concentrates contain oxidized vitamin A, which is biologically inactive, possesses unselective absorption at 328m μ and gives a purple colour with antimony trichloride, and that this oxidized vitamin A is responsible for the reduced value of both factors. Until some method can be found of correcting for this irrelevant absorption and chromogenic activity, the results obtained for the vitamin A content of oils and concentrates must be accepted with reserve and should be supported, when necessary, by biological assays.

Table V. *Relation between biological assay, $E_{1\text{ cm.}}^{1\%}$ and Carr-Price values obtained by different workers*

Worker and material	Biol. assay	Biol. assay	Carr-Price value
	$E_{1\text{ cm.}}^{1\%}$	Carr-Price value	$E_{1\text{ cm.}}^{1\%}$
Carr & Jewell [1933]:			
Concentrate	1250	26	49
Lathbury [1934]:			
Oil X (U.S.P. ref. oil)	1412	44	32
Oils, various	1090-1700	40-45	—
Concentrates, various	880-1450	20-25	—
Hume & Chick [1935]:			
Oil X (U.S.P. ref. oil)	2080	—	—
Oil Y	1430	—	—
Oil Z (subsidiary standard)	2380	—	—
Conc. C	1300	—	—
Morgan <i>et al.</i> [1935]:			
Oil X (U.S.P. ref. oil)	1800	42	42
Cod liver oils, various	1180-1450	27-33	42-48
"Crude" cod liver oil	1120	21	53
Halibut liver oil	1140	21	52
Tunny liver oil	1020	20	52
Liver oil from <i>Thyrsites atun</i>	1650	27	61
Mammalian liver oil	680-1160	17-20	34-57
Commercial concentrates (2)	680, 960	15, 16	44, 60
Robinson, present work:			
Rich concentrate	1060	21.5	52
Oxidation product I	950	22.5	42
" II	650	20.5	31
" III	190	10	18

SUMMARY

A vitamin A concentrate was found to take up 6% of its weight of O_2 when heated in O_2 for $5\frac{1}{2}$ hr. at 100° . After this treatment the ultraviolet absorption had fallen to a low figure, the iodine value and acetyl value had decreased and a small amount of aldehyde had been formed; the product contained no peroxide. The biological activity was small compared with that of the original concentrate. Another concentrate, oxidized in stages by heating in air for varying lengths of time, gave a progressive decrease in iodine value and acetyl value, and a progressive increase in O_2 content, saponification value, aldehyde and ketone content and molecular weight. The biological activity, $E_{1\text{ cm.}}^{1\%}$ 328m μ and Carr-Price value decreased but not at the same rate. The decrease in biological activity measured by bio-assay was more rapid than the decrease in biological activity calculated from the physical measurements by the use of the factors 1600 and 31. The data indicate that the hydroxyl group of vitamin A is oxidized to aldehyde and the unsaturated linkages to peroxides; these are immediately destroyed, first by disruption to form aldehydes and ketones, and secondly, by reaction with an unsaturated linkage to give polymerides. The presence of oxidized vitamin A in concentrates (and in stale fish liver oils) is believed to be responsible for the low values obtained by many workers for the ratio biological assay/ $E_{1\text{ cm.}}^{1\%}$.

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