CLVI. THE ABSORPTION SPECTRA OF THE MIXED FATTY ACIDS FROM COD-LIVER OIL.

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A SURPRISING difference between the absorption spectrum of cod-liver oil itself and that of the mixed fatty acids derived from it has been recorded by Heilbron and Morton and their co-workers. Cod-liver oil itself [Morton and Heilbron, 1928] was found to have relatively low absorption ($E_{1\text{ cm.}}^{1\%} = 1.2$ for a good oil) in the ultra-violet, characterised by the broad unbroken band of vitamin A at 3280 Å. The mixed acids and unsaponifiable matter, on the other hand [Gillam *et al.*, 1931], as obtained by acidification and ether extraction of the hydrolysate, had so intense an absorption ($E_{1\text{ cm.}}^{1\%}$ at 2700 Å. = 250, at 2300 Å. = 190) as to obscure the presence of the admixed vitamin A. The absorption moreover was characterised by fine structure showing not only a band at 2300 Å., which these workers state is shown by a wide range of unsaturated substances, but also no less than 9 narrow absorption bands at 3920, 3750, 3500, 3300, 3160, 3020, 2810, 2700 and 2590 Å. Since the re-esterification of the acids with methyl alcohol or glycerol did not lead to a reversion to the weak absorption shown by the original oil, the change was obviously irreversible, and it was suggested, as the simplest explanation in consonance with all the facts then observed, that under the conditions of hydrolysis a substance (or substances) accompanying vitamin A gives rise to acid decomposition products which display intense selective absorption.

In connection with joint work with the National Institute for Research in Dairying we recently had occasion to carry out a spectroscopic examination on a sample of cod-liver oil which was to be used for feeding to cows. As a result of this work we have encountered a phenomenon which appears to have been overlooked by the Liverpool workers, and which may help to some extent in explaining their observations. According to our experiments the duration of the saponification process exerts such a profound influence upon the absorption spectrum of the acids as to make data obtained under uncontrolled conditions of saponification almost meaningless.

EXPERIMENTAL.

Throughout the present work a Hilger Type E 3 Quartz Spectrograph was used. Specially purified cyclohexane (B.D.H.) was used as solvent.

The ultra-violet absorption of the mixed fatty acids obtained from cod-liver oil by brief saponification. The oil first examined was a crude specimen as used for veterinary purposes. 5 g. of this oil were saponified by heating for about 1 minute with 2.2 cc. of saturated aqueous KOH and 10 cc. of ethyl alcohol. (These

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proportions were used in all the saponifications described below.) The soaps were freed from unsaponifiable matter by extracting 3 times with ether and the free acids obtained by acidification with slight excess of 10 % H_2SO_4 followed by ether extraction. On spectroscopic examination of the mixed acids an absorption maximum was found at 2300 Å., but the value of $E_{1\text{ cm.}}^{1\%}$ was only 7.7.

Since this value was only one twenty-fifth as great as that found by Gillam and his colleagues the possibility occurred to us that the oil might be an abnormal specimen. We therefore repeated the experiment using Engelberg's Norwegian cod-liver oil, a well-known medicinal brand. $E_{1\text{ cm.}}^{1\%}$ at 2300 Å. for the mixed acids was 6.25, a slightly lower value than that for the crude oil.

Absorption of the mixed fatty acids obtained after prolonged saponification. The Engelberg oil, which we used in all subsequent experiments, was next saponified by boiling with the alcoholic KOH on a water-bath for $4\frac{1}{2}$ hours, the unsaponifiable matter then being removed by two extractions with ether and the free acids obtained in the usual way. $E_{1\text{ cm.}}^{1\%}$ 2300 Å. was now found to be 62.5, having thus increased tenfold as the result of the prolongation of the saponification.

The effect of potassium hydroxide on the absorption of the mixed fatty acids. Since Gillam and his colleagues suggest that the high absorption observed by them in the mixed acids may be due to acid decomposition products, presumably derived from the non-saponifiable residue, our next step was to examine the effect of boiling with alcoholic KOH on mixed acids freed from non-saponifiable material. In a preliminary experiment the acids from Engelberg cod-liver oil, $E_{1\text{ cm.}}^{1\%}$ 2300 Å. = 6.25, obtained in one of the experiments described above, were boiled with alcoholic KOH for two periods totalling $4\frac{1}{2}$ hours over a total period of 19 hours. As the result of this treatment $E_{1\text{ cm.}}^{1\%}$ 2300 Å. increased to 110.

Fig. 1. Absorption spectra of mixed fatty acids from cod-liver oil.

- A. Mixed fatty acids obtained after saponification lasting 1 minute.
 B. Same acids after treatment with boiling alcoholic KOH for 15 minutes.
 C. Same acids as A, after treatment with boiling alcoholic KOH for 1 hour.
 D. " " " " " " " " $4\frac{1}{2}$ hours.
 E. " " " " " " " " 12 hours.

The experiment was repeated using a further portion of the oil. In this case the unsaponifiable matter obtained after 1 minute's saponification was separated by 3 ether extractions and preserved for examination. The free acids were then redissolved in alcoholic KOH and heated on a water-bath, samples being taken after periods up to 12 hours. The free acids were obtained by the usual procedure and examined spectroscopically with the following results:

Time of heating with alcoholic KOH	1 min.	15 mins.	1 hr.	4.5 hrs.	12 hrs.
$E_{1\text{ cm.}}^{1\%}$ at 2300 Å. for the free acids	7.6	12	22	39	72

The absorption was again greatly increased as the result of heating with the alkali. Moreover in the case of the acids treated for 4.5 and 12 hours discontinuity in the bands was evident, suggesting the presence of fine structure formation. The shapes of the absorption curves obtained are roughly indicated in Fig. 1.

The effect of potassium hydroxide on the absorption of the non-saponifiable residue of cod-liver oil. The unsaponifiable matter obtained from the 10 g. of oil used in the above experiment amounted to about 130 mg. It was divided into two equal portions. One portion was examined spectroscopically and also by the SbCl_3 reaction, without further treatment, the other was boiled for 4 hours with alcoholic KOH and then re-extracted before examination. The following results were obtained:

N.S.R. equivalent to 5 g. C.L.O.

	Wt. of residue mg.	B.U. per mg. (SbCl_3 test)	$E_{1\text{ cm.}}^{1\%}$ at		
			3280 Å.	2600 Å.	2300 Å.
1 min. boiling with alcoholic KOH	70	250	62	17	38
4 hrs. boiling	66	250	69	34	55

Definite increases of absorption at 2300 Å. and 2600 Å. were therefore observed, but the amount either after 1 minute or 4 hours would represent an altogether negligible contribution in attempting to account for the high absorption observed by Gillam and his colleagues in the combined acids and non-saponifiable residue. On the other hand the vitamin A content according to the SbCl_3 test, remained unchanged. Since, moreover, there was no loss in weight outside the range of experimental error as the result of boiling, it appeared probable that the non-saponifiable residue as a whole showed little tendency to form acid decomposition products under the particular treatment adopted, and no attempt was therefore made to isolate acidic products from the aqueous layer.

Absorption spectra of stearic, oleic and linoleic acids. To obtain some clue to the type of acid taking part in the change leading to increased absorption we have examined the absorption spectra of crude specimens of stearic, oleic and linoleic acids both before and after boiling with alcoholic KOH. Results were as follows:

	$E_{1\text{ cm.}}^{1\%}$ 2300 Å.	$E_{1\text{ cm.}}^{1\%}$ 2300 Å. after 4 hrs. heating with alcoholic KOH
	Initial	
Stearic acid	0.9	1
Oleic acid	16.5	18
Linoleic acid	9.6	38

It will be seen that the absorption of stearic acid and oleic acid was unaffected by the treatment with KOH. The absorption of linoleic acid on the other hand, although initially lower than that of oleic acid, was increased about 4 times after heating with alcoholic KOH.

DISCUSSION.

As far as can be judged from experiments on a single typical specimen of cod-liver oil the above work indicates that the mixed acids obtained as the result of a brief saponification possess relatively low absorptive powers, but that on more prolonged heating with alcoholic KOH they undergo some change which results in much higher absorption.

Whether this phenomenon can be regarded as a complete explanation of the observations of Gillam and his colleagues cannot be decided with certainty, since these workers have given no details of their technique in saponification. As evidence in favour of the existence of two separate phenomena, or overlapping phenomena, it might be argued that in our experiments heating for 12 hours with alcoholic KOH produced only about half the intensity of absorption observed by the Liverpool workers, while fine structure was slightly less fully defined. Such differences might lead us to suspect that cod-liver oils differ fundamentally in their behaviour after saponification from specimen to specimen. In our opinion, however, the cause of the divergence is much more likely to be traceable to differences in the technique adopted for the saponification of the oils and the separation of the acids.

In regard to the chemical nature of the change taking place on heating with alcoholic KOH our experiments on stearic, oleic and linoleic acids appear to indicate that the acids involved must possess two or more double bonds, since only in the case of linoleic acid was increased absorption observed as the result of this treatment. Moreover preliminary iodine value determinations, carried out both on linoleic acid and the mixed acids of cod-liver oil, failed to reveal any significant change in the degree of unsaturation as a result of boiling. It seems possible, therefore, that the increased absorption may be related to an isomeric change among the unsaturated linkages of the more highly unsaturated acids.

From a biological standpoint our results may be of interest in opening up the possibility that highly unsaturated acids may exist *in vivo* in two forms, non-absorptive and absorptive. In an extension of joint work on butter [Booth *et al.*, 1933] we have already obtained evidence which suggests strongly that this may be the case. We hope later to examine non-absorptive and absorptive acids with a view to detecting changes in their chemical behaviour, particularly in their ability to form crystalline bromine derivatives and in their oxygen uptake.

SUMMARY.

The mixed fatty acids prepared from a typical cod-liver oil by a brief saponification, removal of the non-saponifiable matter by ether, acidification and ether extraction showed only relatively low absorption in the ultra-violet region without fine structure ($E_{1\text{ cm.}}^{1\%}$, 2300 Å. = 6.5). If however the time taken for the saponification were prolonged, or if the separated acids were boiled with a further supply of alcoholic KOH, the absorption became much more intense ($E_{1\text{ cm.}}^{1\%}$, 2300 Å. after 12 hours = 72) and definite signs of fine structure became evident.

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