IV. SPECTOGRAPHIC DATA CONCERNING VITAMIN A AND LIVER OILS.

By RICHARD ALAN MORTON, ISIDOR MORRIS HEILBRON and ALBERT THOMPSON.

From the Department of Organic Chemistry, University of Liverpool.

(*Received November 21st, 1930.*)

The object of the present work is to place on record a number of spectroscopic observations on liver oils and concentrates. The data are in the first place concerned with the more precise description of tests for vitamin A, in particular with the ultra-violet absorption and the blue colour test. In the second place, we have utilised spectrographic methods for a more penetrating investigation of the wider problem of the constituents of liver oils and concentrates. We hoped in this way to gain some insight into the sequence of changes culminating in the synthesis of fat-soluble vitamins or provitamins in Nature. Although it is too early to estimate to what extent this hope has been realised, a completely fresh aspect of the chemistry of vitamin-bearing oils emerges from the discovery of new and highly characteristic absorption spectra, which cannot be identified with the properties of the major constituents of liver oils.

It has already been shown [Morton and Heilbron, 1928; Drummond and Morton, 1929] that vitamin A is characterised by a broad, intense absorption band with a maximum near 328$\mu\mu$. Further experience on a wide range of liver oils and concentrates serves to confirm this view, whilst independent support is given in the work of Moore [1930], Capper [1930] and Drummond, Ahmad and Morton [1930] on the conversion of carotene into vitamin A *in vivo*.

The number of samples of crude and refined oils and of concentrates which have been examined in these laboratories is now considerable, and although the correlation between the ultra-violet absorption, the antimony trichloride colour test and the vitamin A potency is more firmly established than ever, attention must be drawn to the fact that disturbing factors can modify the results to some extent. Liver oils vary very widely and the spectroscopic tests are not immune from interference. Due allowance must therefore be made for disturbances caused by unusual composition of certain oils.

In collating the data on cod-liver oils the following facts emerge.

Liver oils. (1) The ultra-violet absorption of oils either in thin films or in alcoholic solution is characterised by a single well-defined maximum at 320–330$\mu\mu$. There is some evidence of a slight step-out in the curve near 300–310$\mu\mu$ and in a few oils of very low vitamin A potency narrow bands

between 300 and 350$\mu\mu$ are faintly shown. A careful examination of the whole of the data, however, satisfies us that the true vitamin A ultra-violet absorption band is in a high degree continuous and free from fine structure.

The vitamin A potency as measured by absorption varies over a somewhat wide range. For a 1 cm. layer of a 1 % solution in alcohol E ($\log I_0/I$, where I_0 = intensity of incident light, I = intensity of emergent light) varies for "genuine refined" cod-liver oils from 0·2 to 2·0. The majority of the oils lie within the limits 0·5 to 1·6. (Carr and Price units are approx. 12 times the above.)

A small fraction of the observed absorption at 328$\mu\mu$ is not due to vitamin A, but to other constituents of the oils. In order therefore for the spectrographic method to be used as a quantitative measure of vitamin A potency, a correction must be applied. For pure weakly coloured cod-liver oils it is generally satisfactory to subtract 0·2–0·3, whilst highly coloured oils of the cattle feeding variety require a somewhat larger correction.

(2) The persistence (*i.e.* $E_{max} - E_{min}$) of the ultra-violet band is variable. This undoubtedly arises from the fact that some fairly absorbent substance other than vitamin A may be present in genuine cod-liver oils, the proportion being liable to considerable variations. The ultra-violet absorption spectrum of any liver oil can therefore be regarded as fixing an upper limit to its vitamin A potency.

(3) Nearly every cod-liver oil exhibits selective absorption in the region 260–295$\mu\mu$. Occasionally it has been possible to record some degree of resolution in this region but for the vast majority of cases only an inflexion near 280$\mu\mu$ can be obtained. No clear parallelism between the absorption at 280$\mu\mu$ and vitamin D potency has been traced, nor has it been possible to associate the absorption with the ergosterol content.

(4) With a few samples of fish-liver oils other than cod-liver oil the absorption at 328$\mu\mu$ is very high but the band appears only as an inflexion. The band is partially masked by an intense abnormal ultra-violet absorption of unknown origin. If however the vitamin A is destroyed by irradiation or aeration and the absorption spectrum is redetermined it is found that the new curve is consistent with the removal of a substance exhibiting the normal vitamin A band.

(5) Spectroscopic examination of the blue solutions with antimony trichloride discloses a maximum which is normally within the limits 604–608$\mu\mu$. Those samples of cod-liver oil which show the 328$\mu\mu$ ultra-violet band clearly and with high persistence also give a blue colour characterised by a single sharp band at 604–608$\mu\mu$ and little or no selective absorption elsewhere in the visible. This class comprises the majority of pale medicinal cod-liver oils and the parallelism between blue colour and ultra-violet absorption is well marked without the introduction of correction factors. With crude cod-liver oils of high potency it is frequently found that the blue solutions exhibit additional selective absorption in the region 565–585$\mu\mu$. Normally this band

appears as a secondary maximum of smaller intensity than the band at 604–608 $\mu\mu$. In one or two samples of cod-liver oil and in a few samples of shark-liver oil the band in the region 565–585 $\mu\mu$ definitely predominates over the normal band. Even in such cases however the 328 $\mu\mu$ band is shown clearly with alcoholic solutions of the original substance; its persistence is unusually low, indicating the presence of additional ultra-violet absorption possibly related to the abnormal colour reaction referred to above.

Further work will be necessary before a decision can be arrived at as to whether the abnormal results are due to a mere displacement of the normal band in the colour test owing to some unusual property of the oil (*e.g.* high content of unsaturated free fatty acid) or to some totally new constituent.

Concentrates. When liver oil concentrates are examined, the blue solutions are found to be characterised by a sharp band with its maximum at 620–624 $\mu\mu$, the intensity varying directly with the intensity of the ultra-violet band at 328 $\mu\mu$ in the original material. The blue solutions with many concentrates also show a less intense band with a maximum at 582–593 $\mu\mu$; when the quantity of concentrate used is sufficient to give very deep blue colours, additional maxima have been seen at 643 and 697 $\mu\mu$ with two separate samples. In the two richest concentrates (from different oils) which we have as yet studied (Carr and Price blue values 14,000 and 9,400 respectively) both the broad continuous ultra-violet band with maximum at 328 $\mu\mu$ and the 624 $\mu\mu$ blue band were clearly exhibited at roughly 1200 and 800 times the intensity shown by a typical cod-liver oil. It is noteworthy that with these concentrates no other maxima were shown.

If concentrates rich in vitamin A are diluted with an inactive oil (*e.g.* seal oil) the quantities being so adjusted that the potency of the product is of the same order as that of cod-liver oil, the absorption maximum in the colour test reverts from 620–624 $\mu\mu$ to the normal position for a cod-liver oil, namely 604–608 $\mu\mu$.

As a result of various chemical treatments which cause decomposition of the material responsible for the characteristic ultra-violet band (328 $\mu\mu$) shown by fish-liver oil concentrates, we have repeatedly observed absorption maxima near 394, 370, 350 and 334 $\mu\mu$. Concomitantly, the concentrates cease to exhibit the 620–624 $\mu\mu$ band with antimony trichloride. The capacity to give a blue colour has not however entirely disappeared for the relatively weakly coloured solutions show a band at 583 $\mu\mu$. Similarly, when a sample of unsaponifiable matter from sheep-liver fat was left in contact with the air for some weeks and extracted with a small quantity of heptane, the extract no longer showed the 328 $\mu\mu$ band but exhibited the same maxima at 394, 369, 349 and 334 $\mu\mu$ (Fig. 1) as were shown by the treated fish-liver oil concentrates. On removal of the heptane and addition of the antimony trichloride reagent a blue solution exhibiting a single band at 583 $\mu\mu$ was again obtained.

At the time that the above observations were made, it was already clear from the work of Drummond and Baker [1929] and from our own experience

that there was little prospect of the direct isolation of vitamin A in a state of purity from liver oils. It was also becoming evident that while the hydrocarbon carotene was not itself vitamin A [Dulière, Morton and Drummond, 1929] it was enormously potent in promoting growth [Euler, Euler and Hellström, 1928; Moore, 1929; Collison, Hume, Smedley-MacLean and Smith, 1929]. The direct attack on vitamin A was in a position of stalemate and the indirect attack through carotene seemed promising. The new absorption bands referred to above seemed worth further study since they might throw light either on the substance from which vitamin A is made or on substances derived from the breakdown of the vitamin molecule.

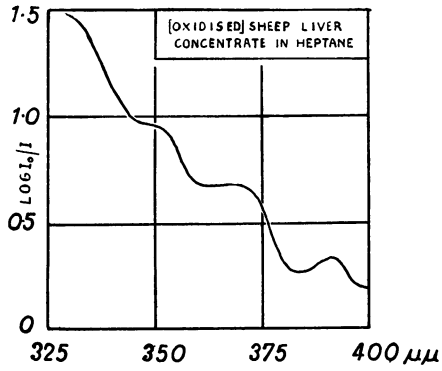

Fig. 1.

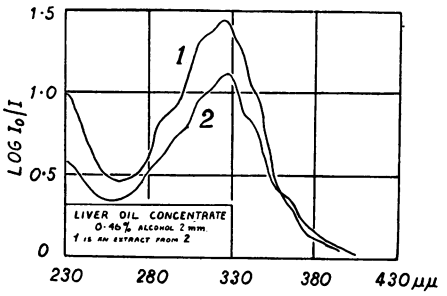

Fig. 2.

We chose first to investigate the decomposition products of the vitamin. Before however reporting on the absorption spectra of concentrates from which the typical ultra-violet band had been removed, it is necessary to reconsider this band. So far, it has been described as a broad continuous band having no recognisable fine structure.

Using a concentrate 200 times as potent as cod-liver oil we obtained indications of fine structure (Fig. 2). Attempts to increase the degree of resolution either by replacing alcohol with hexane as the solvent or by the use of still richer concentrates proved fruitless. As a result of experiments involving distribution between partially miscible solvents a very considerable increase in potency was obtained in one fraction but the indications of fine structure remained quite indefinite. As it was further found that the degree of resolution was less rather than greater, when a concentrate 1000 times as rich as cod-liver oil became available (Fig. 3) it was resolved to proceed on the two assumptions that the vitamin A band was really highly symmetrical and that the indications of fine structure arose from other substances, the relationship of which to vitamin A was unknown.

On this basis the next step was clearly to effect the removal of the 328 $\mu\mu$ band by decomposition of the vitamin in order to see if any fine-banded absorption could be revealed after eliminating the obscuring effect of the main broad band. Numerous trial experiments were made, but only one

need now be mentioned, namely the evaporation of the concentrate with strong alcoholic sodium ethoxide. An extract of this product disclosed on spectrographic examination (*a*) the absence of the 328 $\mu\mu$ band, (*b*) a diminution in intensity of absorption and (*c*) an increase in selectivity. With antimony trichloride a comparatively feeble blue colour was given, but the 624 $\mu\mu$ band was absent, the only absorption band present having its maximum at 583 $\mu\mu$.

As this experiment had proved fruitful, the work was repeated on a larger scale. For this experiment a neutral cod-liver oil concentrate (obtained from Messrs Lever Bros., Ltd.) having a vitamin potency of 2400 Carr and Price units was employed. As a preliminary measure, the saponification equivalent of the concentrate was determined, using a large excess of reagent and applying heat for a longer period than usual. The material was found to be practically

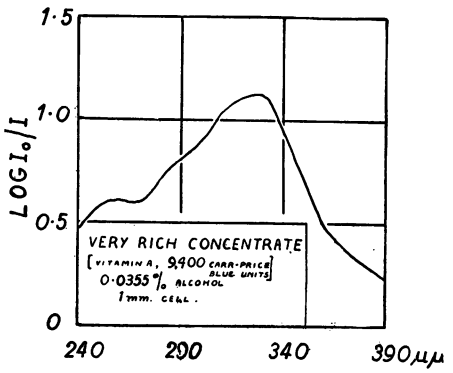

Fig. 3.

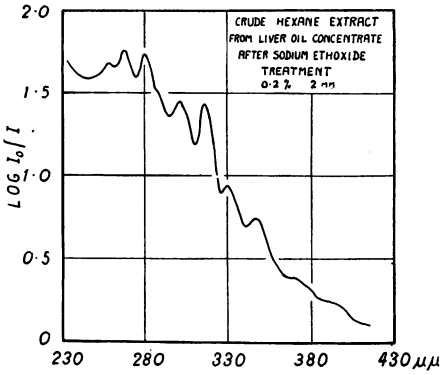

Fig. 4.

free from saponifiable matter (saponification equivalent, 19,000). A further portion of this concentrate was treated as follows. Sodium (25 g.) was dissolved in alcohol (250 cc.) and the solution was heated in an atmosphere of nitrogen in an oil-bath for 2 hours at 150–160° with the concentrate (25 g.). The alcohol was removed under reduced pressure, the residue was dissolved in water (500 cc.) and the whole was twice extracted with ether. The ethereal solution was thoroughly washed with water, dried over sodium sulphate, and evaporated, yielding a dark, brown oil.

The colour test with antimony trichloride, however, revealed the presence of unchanged vitamin A and in consequence the above treatment was repeated. The neutral portion so obtained disclosed (as shown in Fig. 4) on spectrographic examination a series of definite maxima near 394, 375, 350, 330, 316, 302 (inflexion 290), 282, 271 and 260 $\mu\mu$. With antimony trichloride only a band at 583 $\mu\mu$ could be recorded. The combined alkaline liquid from the above experiments was acidified with dilute sulphuric acid and the precipitated oil was extracted with ether. The ethereal solution was shaken with dilute sodium carbonate solution to remove acids, leaving in the ether some neutral resinous material. The acids were precipitated from the sodium carbonate solution and obtained as a dark brown oil (about 2 g.). It will be noted

that the weight of acids isolated is between 5 and 6 times in excess of that demanded by the saponification equivalent (assuming saponifiable matter to be cod-liver oil glycerides) and corresponds to approximately 0·06 % of the original cod-liver oil.

The absorption spectrum of these acids is reproduced in Fig. 5; it will be

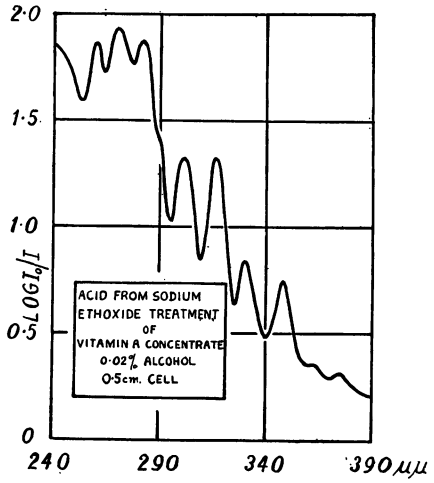

Fig. 5.

observed that this resembles the curve shown in Fig. 4 from which it appears that the characteristic selective absorption is due to acid material. The fact that this is shown in the original so-called neutral portion is due to the solubility of the sodium salts in ether, for by repeated washing of the ethereal solution, first with dilute alkali and then with water, a true neutral material was ultimately obtained in which the fine absorption bands had almost entirely disappeared; with antimony trichloride a violet-blue solution was obtained exhibiting a maximum of considerable intensity at 583$\mu\mu$.

Owing to the difficulty experienced in accomplishing complete decomposition of the vitamin in the above experiment, it was decided to work at a somewhat higher temperature and for this the alcoholic sodium ethoxide was replaced by sodium amyloxide. The material employed in this case was a cod-liver oil concentrate obtained from Messrs J. Nathan and Co., Ltd. The vitamin A potency was of the same order as that of the Lever extract, but the saponification equivalent in this case was 3900. The material was accordingly re-saponified with 5 % alcoholic potash; the product obtained had then a saponification equivalent of 8200, and was treated with sodium amyloxide in the following manner.

Sodium (12·5 g.) was dissolved in distilled amyl alcohol (125 g.), contained in a 1000 cc. round-bottomed flask fitted with a reflux condenser, and the solution was heated in an oil-bath at 180° in a current of nitrogen. The vitamin concentrate (25 g.) was added, and the oil-bath was kept at 180° for 2 hours,

after which the amyl alcohol was removed as far as possible under reduced pressure. The solid residue was then cooled in an atmosphere of nitrogen and heated with distilled water (250 cc.) until the sodium amyloxide had been decomposed. The product was extracted with ether (1000 cc.), acetone being added to break the emulsions formed. The ethereal extract was repeatedly washed with water, and the washings were combined with the first aqueous extract. This was again extracted with ether until the soaps were entirely freed from neutral material. The alkaline solution was acidified with dilute sulphuric acid, saturated with salt and extracted with ether. The extract was dried over anhydrous sodium sulphate, giving on evaporation the acid portion (3 g.) again in amount considerably in excess of that calculated (0·9 g.) from the saponification equivalent taken as cod-liver oil glycerides. The spectrographic examination of the acid material gave the curve shown in Fig. 6,

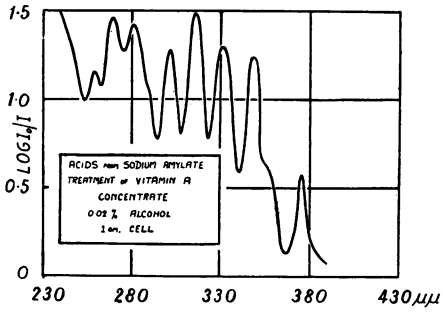

Fig. 6.

revealing the same series of bands as were obtained from the sodium ethoxide treatment. The intensity distribution differed in the two cases and comparison of the respective curves makes it highly probable that the acidic material is heterogeneous, consisting of at least three different selectively absorbing acids. The group of bands at 260, 271 and 282 $\mu\mu$ appears to be due to one substance, the bands at 301 and 317 $\mu\mu$ to another, those on the long wave side of 317 $\mu\mu$ to at least one other acid, selective absorption beyond 260 $\mu\mu$ remaining to be accounted for.

The production of these strongly absorbing acids next led us to examine spectrographically the total acids obtained by the ordinary saponification of cod-liver oil. We found here that the same type of absorption was shown by these acids [for curves see Gillam *et al.* 1931, pp. 34, 35], the ten characteristic bands being equally well-defined. At first sight it would thus appear plausible to suggest that the selective absorption finds its origin in the ordinary fatty acids; this view is however untenable for it fails to account for the absence of this complex absorption from cod-liver oil itself. Calculation of the intensity of the vitamin A band as compared with that of the acids rules out completely the suggestion that the acid bands are masked by the 328 $\mu\mu$ band. Taking the intensity of absorption of a 1 % solution of cod-liver oil using a 1 cm. layer as unity, the intensity of absorption shown by the acids is about 50 times

as great. Masking being therefore eliminated an alternative hypothesis must be sought. Attention may be drawn to two significant points. In the first place it is highly improbable that the usual process of saponification would fail to hydrolyse any materials present in the fat as ordinary glycerides, and in the second place the amount of acidic material obtained from the concentrates by treatment with sodium ethoxide or amyloxide is largely in excess of that calculated from the saponification value.

The highly absorbing acids are therefore unlikely to be simple fatty acids and probably result from drastic and irreversible changes. The richest concentrates of vitamin A after treatment with sodium amyloxide did not exhibit the ten narrow bands, the decomposition products showing only one maximum at 276$\mu\mu$ (Fig. 7). The acids cannot possibly be accounted for as arising solely

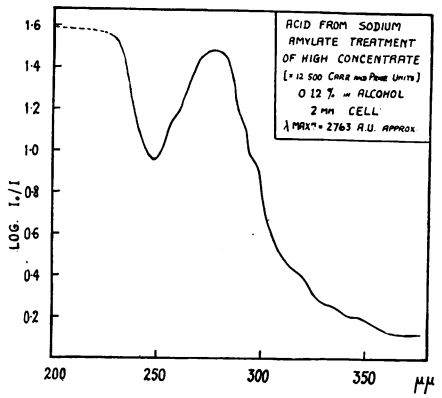

Fig. 7.

from the decomposition of vitamin A because the intensity of the new system of bands is no greater in the preparations from concentrates than in the total acids resulting from the ordinary saponification process, *i.e.* the accumulation of the vitamin in the concentrate is not accompanied by a corresponding increase in the amount of the highly absorbing acids. This experiment seems definitely to eliminate vitamin A itself as a sole contributory factor in the formation of the highly absorbing acids. The retention, in all but the highest concentrates, of material capable of yielding the acids possibly indicates that the bands are associated with either the later stages in the synthesis of vitamin A or the earlier stages of its utilisation in the organism. The band at 276$\mu\mu$ may however well arise as a direct decomposition product of the vitamin, a suggestion supported by the appearance of such a band as a result of the photochemical destruction of vitamin A [Morton and Heilbron, 1928]. Control experiments on the action of sodium amyloxide on both ergosterol and dehydroergosterol have been carried out, and show that decomposition of these substances does not in any way account for the appearance of the absorbing acids.

We are therefore left to conclude that cod-liver oils contain appreciable

quantities of highly transparent substances which are decomposed during the process of saponification giving acids characterised by intense selective absorption. A much smaller quantity of not dissimilar, relatively diactinic material remains undecomposed and passes into the unsaponifiable extract. Drastic treatment with alkali is necessary before this residual, possibly more resistant, material is attacked, but when, as with sodium ethoxide or amyloxide, this occurs the acids produced are of the same type as before.

It is impossible not to be struck by a qualitative similarity between some of the groups of bands and the spectra of ergosterol and dehydroergosterol [Heilbron, Johnstone and Spring, 1929], and the fact that carotene shows a similar set of bands displaced into the visible [Dulière, Morton and Drummond, 1929]. The reappearance of well-resolved bands, again in groups of three, may well indicate that the acids are not unrelated to substances either of the poly-ene or sterol type.

Summary.

1. Precise spectroscopic data regarding the vitamin A ultra-violet absorption band are recorded.

2. The vitamin A band at 328$\mu\mu$ is found to be free from fine structure.

3. Nearly all cod-liver oils exhibit selective absorption in the region 260–295$\mu\mu$.

4. Spectroscopic examination of the blue solutions obtained with antimony trichloride disclose, with cod-liver oils giving a clear ultra-violet band at 328$\mu\mu$, a single sharp band at 604–608$\mu\mu$.

5. With crude cod-liver oils of high potency additional selective absorption between 565–585$\mu\mu$ is frequently observed in the blue solution.

6. The blue solutions given with concentrates have the main band at 620–624$\mu\mu$ and in many cases show a less intense band at 582–593$\mu\mu$.

7. Vitamin A is decomposed on treatment with sodium ethoxide. Concentrates so treated yield acids characterised by a series of well defined absorption bands with maxima near 394, 375, 350, 330, 316, 302, 282, 271 and 260$\mu\mu$.

8. Similarly absorbing acids are produced by the ordinary saponification process, but evidence is adduced showing that these acids cannot be ordinary fatty acids and that they are not present as simple glycerides in the oil itself.

We desire to express our thanks to Messrs Lever Brothers, Ltd., and Messrs Joseph Nathan and Co., Ltd., for material used in this investigation, and also to the Department of Scientific and Industrial Research for a grant to one of us (A. T.).

REFERENCES.

Capper (1930). *Biochem. J.* **24**, 453.
Collison, Hume, Smedley-MacLean and Smith (1929). *Biochem. J.* **23**, 634.
Drummond, Ahmad and Morton (1930). *J. Soc. Chem. Ind.* **49**, 291 T.
—— and Baker (1929). *Biochem. J.* **23**, 274.
—— and Morton (1929). *Biochem. J.* **23**, 785.
Dulière, Morton and Drummond (1929). *J. Soc. Chem. Ind.* **48**, 316 T.
Euler, Euler and Hellström (1928). *Biochem. Z.* **203**, 370.
Gillam, Heilbron, Hilditch and Morton (1931). *Biochem. J.* **25**, 30.
Heilbron, Johnstone and Spring (1929). *J. Chem. Soc.* 2248.
Moore (1929). *Biochem. J.* **23**, 803.
—— (1930). *Biochem. J.* **24**, 692.
Morton and Heilbron (1928). *Biochem. J.* **22**, 987.