Absorption Spectra Of Lipids And Their Application To Metabolic Studies*

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Abstract

Absorption curves are given for some purified fatty acids, commercial oils and tissue lipids. There is a specific absorption band for fatty acids at about 2300 A.U. Many lipids have absorption coefficients far too high to be accounted for by the well recognized fatty acids. It is shown that this is not related to degree of unsaturation, but is best accounted for by conjugation of double bonds. Hence absorption spectra serve as one of the best quantitative measures of conjugation in its various forms. In metabolism the rat attacks only one of the bonds in eleostearic acid at a time, leaving the other two for a sufficient period to be measured spectroscopically.

IN this paper methods are described and the first results are given of an extended study of lipids by photoelectric spectrophotometry. Already, other workers have established the usefulness of absorption spectra in identification of unknowns and the quantitative analysis of known organic compounds. By application of similar technique to the common lipids it has been possible to establish the presence of as yet unidentified lipids in tissues and to follow the course of utilization of known lipids by the rat.

Methods

The photoelectric spectrophotometer is similar in design to the apparatus described by Hogness, Zscheile, and Sidwell (9).

The spectral region is isolated from the continuous spectrum by a Zeiss Fixed Arm spectroscope (deviation 90°), used as a monochromator. Referring to wave length 4861 A.U., the effective aperture is F/6.4 for the entire instrument. The error of the wave length drum is less than 1 A.U. at 2150 A.U. and 3 A.U. at 7800 A.U. Although this instrument is equipped with achromatic lenses, a slight adjustment is necessary to correct for chromatic errors.

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A DuBridge and Brown circuit is employed to amplify the photoelectric current. In this circuit an F.P. 54 vacuum tube (General Electric) is employed for the amplification of the current. The photoelectric current is passed through a high resistance Rg (1×10^{11} ohm), and the potential across this resistance is impressed on the grid of the amplification tube. The amplified current is measured by a galvanometer (Leeds Northrup Company: 2500-b, period 6 sec.). The sensitivity of the galvanometer is controlled by an Ayrton shunt. The photocell is a cesium oxide gas filled type, fitted with internal and external grounds.

With proper electro-magnetic shielding a stable voltage sensitivity of 225,000 mm. per volt and a current sensitivity of 1×10^{-16} amperes per mm. is attained. These sensitivities are for 4 meter scale-galvanometer distance with a galvanometer sensitivity of 0.6. The galvanometer drift is less than 0.5 cm. per hour with oscillations 1 mm. Usually 40-50 seconds are sufficient for the measurement of two absorption coefficients. The cell carriage which holds three absorption cells (Hilger type 291) is placed behind slit No. 2. Thus, from four transmission measurements, two absorption coefficients may be calculated.

The over-all error for the apparatus, including fluctuations in light source, amplification tube, photoelectric cell, and galvanometer, is less than ± 0.25 per cent for the visible region and less than ±0.35 per cent for the ultraviolet region. The larger error for the ultra-violet region is due to fluctuations in the light source.

Calibration: The following tests were made to standardize the apparatus:

A. Known voltages were impressed on the grid of the amplification tube and the deflections of the galvanometer were observed. Direct proportionality was found.

B. The proportionality between the response of the galvanometer and the intensity of the incident light on the photoelectric cell was determined by the interposition of a series of calibrated screens. These screens were calibrated by a thermopile. The transmission values obtained by the photoelectric apparatus agreed (error ± 0.5 per cent) with those obtained by the thermopile.

C. As a check on the total error of the apparatus, light source, and observer, the absorption coefficients were measured on a standard solution consisting of equimolar concentrations of potassium chromate and copper sulfate in 2N. ammonia solution. The absorption spectrum of this standard in the visible region agreed with the published values of Weigert and Smith. In the ultraviolet region our photoelectric data was slightly lower than that of Weigert.

D. Calibrated pyrex filter: Through the courtesy of the Bureau of Standards a calibrated filter* was obtained as an additional check in the ultraviolet. The transmission values obtained by the apparatus is compared with those obtained by the Bureau of Standards in Table I.

TABLE I

A comparison of transmission values of a pyrex filter as measured by the thermopile (Bureau of Standards) and the photoelectric apparatus.

Method of Measurement	Wave lengths in A.U. 3020 %	3130 %	3340 %
Thermopile	29.5	54.5	86.0
Photoelectric	28.9±0.2	54.6±0.2	85.9±0.1

*The use of a calibrated filter is now considered the most reliable method for checking the total error of the spectroscopic apparatus.

Experimental

The Fatty Acids: As a preliminary to the lipid studies, absorption curves were determined on all of the saturated fatty acids with an even number of carbons from acetic acid to stearic acid. In this study samples of fatty acids were obtained from many sources and the curves were determined before and after purification. Diethyl ether was used as the solvent in all work reported here.

The absorption coefficients are largest for acetic acid and decrease as the carbon chain lengthens. With some of the fatty acids a considerable range of variation was obtained, but with other acids the range was small.

When E (molecular extinction coefficient) is calculated from α all acids approach an average value of 19 with a range of from 15 to 22.

TABLE II

Molecular extinction coefficient (E) at 2300 A.U. of the saturated fatty acids calculated from the mean values of all determinations.

Acid	Mol. wt.	α	E
Acetic	60	2.5	15
Butyric	88	2.2	18
Caproic	116	1.8	21
Caprylic	144	1.5	22
Capric	172	1.3	22
Lauric	200	.9	18
Myristic	228	.8	18
Palmitic	256	.7	18
Stearic	284	.65	18
Average			18.8

This is in close agreement with the values given by Ramart-Lucas, Biquard and Grunfeldt (14), which range from 16 to 25. These workers attribute this range in value to impurities and experimental error, and point out the necessity for great care in purifying the acids. However, they do not find the specific maxima at 2300 A.U. but only a general absorption.

In this laboratory five samples of stearic acid were recrystallized from alcohol at intervals of several days. The α at 2300 A.U. ranged from 0.63 to 0.82 with an average of 0.71.

The present work supports the view that the absorption at 2300 A.U. is proportional to the concentration of carboxyl groups and independent of the length of hydrocarbon chain. Further attempts will be made to explain the fluctuation thus far encountered in the absorption of saturated acids. Values for the unsaturated fatty acids will be reported in the near future.

The Vegetable Oils: Determinations of the absorption curves of linseed oil, corn oil, olive oil, coconut oil, perilla oil, poppy seed oil and tung oil have been made. The study includes a comparison of several samples of commercial linseed oil, corn oil and olive oil from different sources.

Fig. 2 illustrates typical curves

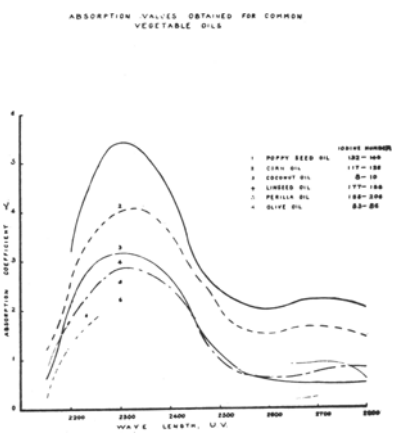

FIGURE 2.

obtained with the different oils. No correlation was found to exist between the maximum α and the iodine number of the oil. The chemical analyses of these oils furnish no clue as to the reason for the differences obtained. Moore (12) has recently reported $E^{1\%}_{1\,cm}$ at 2300 A.U. for the mixed fatty acids of a number of vegetable oils and animal fats. Only in the case of linseed oil is there close agreement with the values given in Fig. 2. He finds no relation between iodine number and absorption. Gillam *et al.* (8) report absorption values for three oils, all of which are markedly higher than those in Fig. 2.

Fig. 3 illustrates the similarity existing between samples of commercial oils from different sources. Of the three oils charted, the linseed oil and olive oils were obtained by cold press methods, while the corn oils were probably the products of the common hot press technique. The consistent reproducibility of α indicates that these values are specific for each oil and not due to fortuitous impurities. Particularly striking is the agreement between commercial cold press linseed oil and oil extracted in this laboratory by grinding flax seed

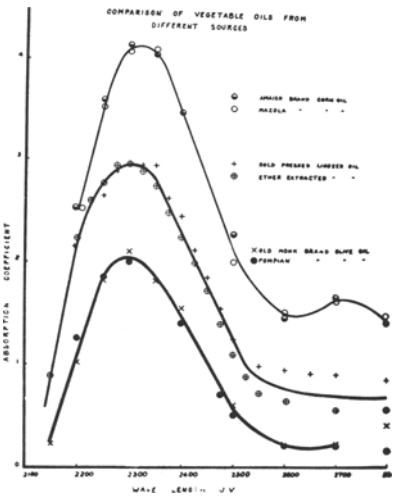

FIGURE 3.

with pure ether at room temperature. The ether was removed *in vacuo*. Experiments with ether extracted corn germ indicate that some changes may occur in corn oil during the manufacturing process.

Tung oil has not been included in Fig. 2 because it differs so markedly from all the other oils studied. With the exception of tung oil the highest values for α has always been found within the range between 2250 A.U. and 2350 A.U. With tung oil,* however, the maximum value for α is 1400 at 2700 A.U. (in diethyl ether). The peculiarity of tung oil is due to the presence of α- and β-eleostearic acids, which are apparently unique among the known fatty acids. Dingwall and Thomson (7) have given absorption curves for α- and β-eleostearic acids in hexane. $\alpha = 1580$ at 2700 A.U. for their sample of oil.

Mixed Fatty Acids: After some experimentation a two minute saponification according to Dann and Moore (5) was adopted. This treatment, followed by rapid ether extraction to remove unsaponifiable material, immediate acidification with HCl and washing, was found to lower slightly the maximum α for the fatty acids as compared

*We are indebted to Mr. B. F. Williamson, Gainesville, Florida, for samples of perfectly fresh tung oil.

with that of the original oil. In these studies all attempts at purification have resulted in a lowering of the maximum value for α. Oxidation always causes a gradual increase. We therefore feel that the lower values probably most nearly represent the true value for unchanged lipids. Four washings with distilled water to remove traces of HCl were found to produce optimum results. The solvent was finally removed *in vacuo* at low temperature. The absorption values obtained for the mixed fatty acids at 2300 A.U. are compared with the original oil in Table III.

TABLE III

Absorption values for oils and their mixed fatty acids.

	α at 2300 A.U.
Corn oil	4.1
Coconut oil	3.2
Linseed oil	2.9
	α at 2300 A.U.
Corn oil fatty acids	3.3
Coconut oil fatty acids	2.8
Linseed oil fatty acids	2.4

Since the change is so uniform for the oils, it would seem that about the same amount of absorbing material was removed from each. This might be in the unsaponifiable fraction. Since Henri found slightly lower values for esters than for free acids, it does not seem probable that the glyceride structure itself increases absorption.

The Animal Fats: Only a few animal fats have been studied. These are pig liver lipids, pig leaf fat, pig blood lipids, bovine blood lipids, human breast milk fat, bovine milk fat and rat milk fat. The lipids of solid tissues were extracted by repeated grinding in a mortar with pure quartz sand in purified alcohol and ether at room temperature. The extracts were filtered and concentrated at low temperature *in vacuo*. The residue was extracted with purified ligroin, B. P., 30-35°, centrifuged and the true lipids recovered from the solvent *in vacuo*.

The bovine blood was collected from the jugular vein with large needle and syringe. No visible hemolysis occurred. The serum was separated by centrifuging and poured into Bloor's mixture of purified alcohol and ether at room temperature. The extract was concentrated and true lipids recovered as described above.

Pig blood serum was collected at the killing room of a large packing house. Appreciable hemolysis occurred because of rough treatment during collection.

Only the simplest fractionations are reported here. The total lipids were separated into neutral fats and phospholipids by pouring a concentrated ether solution into excess of acetone. The precipitate was redissolved in ether and again thrown down in acetone. The usual two minute saponification in alcoholic KOH was used for separation into fatty acids and unsaponifiable matter. There was only a trace of unsaponifiable matter in the phospholipid fraction.

The buttermilk lipids were extracted with alcohol and ether from 10 gallons of freshly churned buttermilk containing about 0.1% butterfat. This study was made because Kurtz, Jamieson and Holm (10) reported about 6 per cent of dicostetrenoic acid in phospholipids extracted from dried buttermilk.

Of these fats, only the pig leaf fat gives a curve similar to those of the vegetable oils. The liver lipids absorb very much more strongly at 2500 A.U. The blood lipids and milk lipids invariably have higher absorption coefficients at 2300 A.U. than do the other lipids studied.

Fig. 4 shows the curves obtained

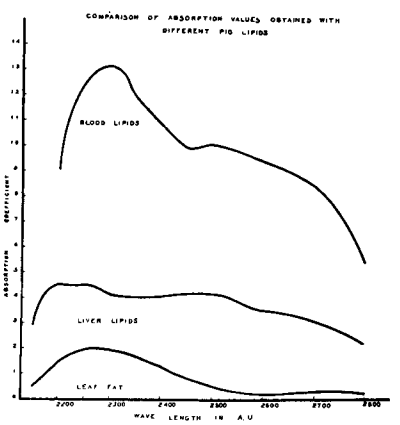

FIGURE 4.

with leaf fat, liver lipids and blood lipids, while Figs. 5, 6 and 7 show the respective results obtained in fractionation studies of pig blood lipids, bovine blood lipids and buttermilk lipids. In every case the phospholipid fatty acids are more absorptive than those from the

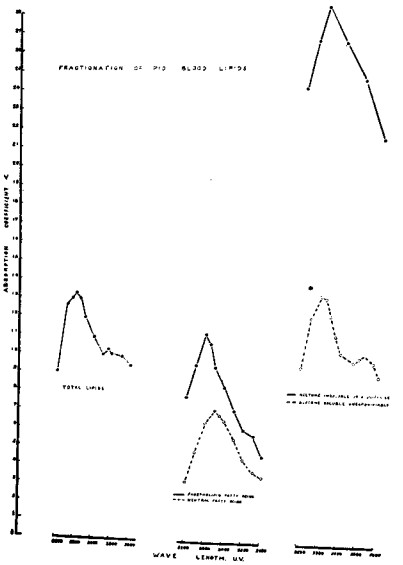

FIGURE 5.

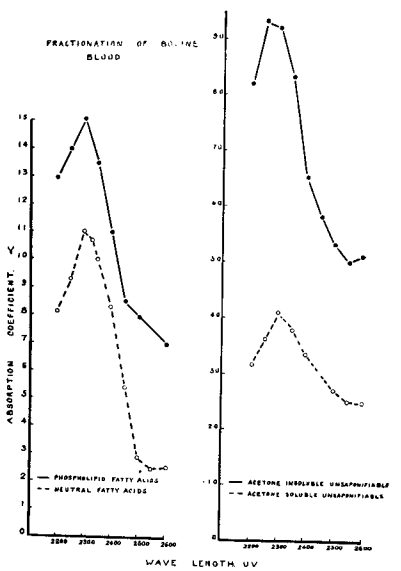

FIGURE 6.

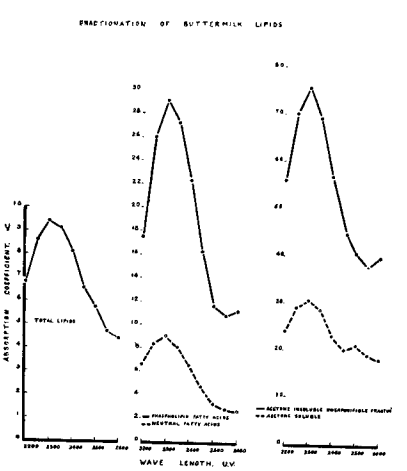

FIGURE 7.

neutral fats. The very high absorption of buttermilk phospholipid acids is most striking and may be due to docosatetrenoic acid.

Peak values of $\alpha = 7.15$ for human breast milk lipids and $\alpha = 19.2$ for rat milk lipids indicate that high values are probably common to all milk fats. The effect of time after parturition is shown in Fig. 8.

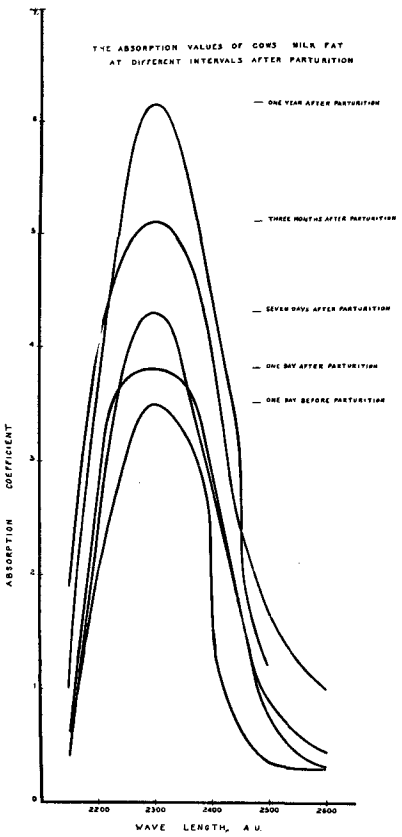

FIGURE 8.

These milk fats were collected during one week from three cows (Nos. 178, 204, 198). All were on the same dry feed and in the same herd. These values are in good agreement with the E_{2300} given by Booth *et al.* (2) for butter from cows on dry feed (winter season).

Discussion: Following the work of Gillam and coworkers (8) who first undertook absorption studies of mixed butter fatty acids, Booth *et al.* (2) showed a large seasonal variation in the specific absorption at 2300 A.U. The $E^{1\%}_{1\,cm.}$ ranged from a low of 5 in late winter to a high of 25 immediately after the cows went on pasture in May. The authors postulated the presence of a highly absorptive acid as yet unidentified, which is most likely made by the cow from ingested fats. Later work by Dann *et al.* (6) showed that the effects of dietary fats decreased in the order: cod-liver oil, sardine oil, linseed oil, and rape oil. Similar increases in absorption produced by prolonged boiling in alkali are likely due to molecular rearrangement and not to oxidation. Recently, Moore (12) has shown that the production of highly absorptive acids with alkali is more or less proportional to iodine number of vegetable oils and that saturated acids and oleic acid are not responsible for the change. Linoleic acid rearranges to an acid strongly absorbing at 2300 A.U., while linolenic acid changes into a solid form with the chief band at 2700 A.U. This new acid is not identical with α- or β-eleostearic acid. It is interesting that acids absorbing at 2300 cure fat deficiency in rats, while the ones absorbing at 2700 A.U. probably do not. α-eleostearate was shown by Burr, Burr and Miller (3) to be non-curative.

Crymble *et al.* (4) in 1911 showed that only compounds with conjugated double bond systems had strong general absorption in the ultraviolet. Conjugation in an aliphatic chain may increase general absorption by 100 times. Thus, crotonic acid is much more active than allyl acetic and enol forms more than keto forms. Morton, Hasson and Calloway (13) have recently extended this work to specific absorption bands. They show that an absorbing entity is made up of two or more simple chromophores such as $c = c$ or $c = o$. Conjugation of two double bonds may increase the absorption maximum by 100 x, and as the number of conjugated double bonds increases the maximum moves to longer wave lengths.

The very strong absorption by the eleostearic acids at about 2700 A.U. is readily accounted for by the presence of three conjugated double bonds (Böeseken, 1). On the other hand, ordinary linolenic acid has no conjugated system and very low absorption. Hulst (15) has likewise shown that while ordinary linoleic acid is very transparent 9-11 linoleic acid has a powerful band at 2300 A.U. Similarly, 2-3 oleic acid absorbs much more than ordinary oleic because of the conjugation $c = c - c = o$.

The high transparency of the mixed fatty acids in many common seed oils and in adipose tissue indicates almost total lack of acids with conjugated systems. On the other hand, the relatively high absorption of mixed lipids from blood and organs and some milks points to the presence of hitherto unrecognized acids. Since a common acid like pyruvic absorbs strongly in this region it is necessary to look carefully for $c = c - c = o$ conjugation as well as $c = c - c = c$. The presence of absorptive aromatic or cyclic compounds would be recognized by absorption in the longer ultraviolet.

By the use of eleostearic acid of tung oil as an indicator in fat metabolism, Miller and Burr (11) have shown interesting differences in the rate of deposit and destruction by several tissues. A possible insight into the mechanism of destruction of eleostearic acid by tissues is given by the gradual disappearance of the band at 2700 A.U. and its replacement by one at 2350 A.U. Böeseken (1) has shown that by successive hydrogenation 10-12 linolic acid is produced from eleostearic acid. Hulst (15) followed the spectral changes during selective hydrogenation of eleostearic acid and found a new band at 2300 A.U. as the one at 2700 A.U. disappeared. He postulates that this is due to the production of 10-12 linolic acid which would absorb almost in the same way as 9-11 linolic. From the great similarity of Hulst's curves to those found for rat tissues it may be postulated that the first stage in destruction of eleostearic acid is the selective hydrogenation of one of the three double bonds.

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