XVII. SPECTROSCOPIC CHANGES IN FATTY ACIDS

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I. CHANGES IN THE ABSORPTION SPECTRA OF VARIOUS FATS INDUCED BY TREATMENT WITH POTASSIUM HYDROXIDE

THE effect of treatment with KOH in increasing the intensity of the absorption spectra of fatty acids was first established by Dann & Moore [1933]. It was shown that the intense fine-structured absorption previously observed by Gillam *et al.* [1931] in the mixed fatty acids of cod liver oil was absent if the acids were prepared by a brief saponification of the oil, but appeared with increasing intensity as the acids were refluxed with hot alcoholic potash. The effect of potash in increasing absorption was confirmed by Edisbury *et al.* [1935] for the acids of various marine oils. Dann *et al.* [1935] found that the absorption was increased not only in marine oils, but also to a lesser extent in two vegetable oils. In their paper evidence was also advanced to show that when the mixed acids of cod liver oil were given to cows in the “pro-absorptive” form, they were secreted in the milk in an “absorptive” form. Although it was undecided whether the changes produced by treatment with potash were identical with those occurring in the course of absorption and secretion by the cow, it seemed probable that in each case isomeric changes in acids having at least two unsaturated linkages were involved. The purpose of this section of the present paper is to record spectroscopic data obtained on oils and fats of various types.

¹ An “absorptive” acid is one showing high absorption in the spectroscopic sense. A “pro-absorptive” acid is one which itself has little specific absorption, but is capable of being converted into one which possesses such absorption, either *in vitro* or *in vivo*. For convenience the absorption which could be developed in a “pro-absorptive” acid by appropriate treatment is termed “potential absorption”.

EXPERIMENTAL

Technique. 5 g. specimens of the oils or fats were saponified by heating for about 1 min. with alcoholic potash (2 g. KOH, 1.4 ml. water, 10 ml. EtOH). The unsaponifiable matter was removed by ether. The soaps were acidified with sulphuric acid (sp. gr. 1.2). The ether layer was washed with water and the ether removed on a water-bath under suction. A portion of the acids was retained for immediate spectroscopic examination. The remainder of the specimen was refluxed, usually for 24 hours, with more alcoholic potash, and the acids were separated as before. The spectroscopic absorption of the mixed acids before and after prolonged treatment with potash was determined using a Hilger Spekker spectrophotometer, H 290. The solvent was usually alcohol, but *cyclohexane* was used occasionally.

The results obtained are shown in Tables I-IV.

DISCUSSION

The nature of the acids undergoing spectroscopic changes. The data on the mixed acids from vegetable oils (Table I) support the previous view that the acids responsible for increased spectroscopic absorption are those possessing two or more unsaturated linkages (polyethylenic acids). The acids from coconut oil, an almost saturated vegetable fat, show very low absorption, which remains low after potash treatment. Only slightly higher absorption was observed in the

Table I. *Vegetable oils and fats*

	Reputed I.V.	$E_{1\text{ cm.}}^{1\%}$ at 230 m μ 1 min. sap.	Remarks	$E_{1\text{ cm.}}^{1\%}$ at 230-235 m μ 24 hr. sap.	Remarks
Coconut oil	8-9.5	1	End abs.	2	End abs.
Palm kernel oil	13-14	9.9	End abs.	5.8	End abs.
Red palm oil	51.5	6.7	Flat end abs.	13	Flat end abs.
Olive oil	79-88	7.5	Flat end abs.	12	Flat end abs.
Arachis oil	83-100	5.5	End abs.	51	Flat band
Wesson cotton-seed oil	108-110	5	Band at 275 m μ , $E_{1\text{ cm.}}^{1\%}$ 6	120	Band
Soya bean oil	122	21	End abs.	120	Band
Linseed oil	171-201	2.4	End abs.	170	Band
Tung oil	150-165	180	Max. at 275 m μ , $E = 1100$	—	—

Reputed iodine values taken from Lewkowitsch, *Laboratory Companion to Fats and Oils Industries*, Macmillan, 1901.

Abbreviations: End abs.=end absorption. Max.=absorption maximum.

Table II

	I.V.	1 min. sap.	Remarks	24 hr. sap.	Remarks
Land mammal fats					
Ox body fat	35-46	7.2	Band 230 m μ	14	Band 230 m μ
Sheep body fat	38-46	33	Band 230 m μ	36	Band 230 m μ
Pig body fat	50-70	23	Flat band 230 m μ	23	Flat band 230 m μ
Human	61.5	12	Band 230 m μ	22	Band 230 m μ
Rat (given diet containing red- palm oil)	—	13	Flat end abs.	36	Flat end abs.
Bird fat					
Duck	58.5	1.8	End abs.	27	Band 230 m μ

Table III. *Marine fat*

	$E_{1\text{ cm.}}^{1\%}$ after 1 min. sap.		Remarks	$E_{1\text{ cm.}}^{1\%}$ after 24 hr. sap.		Remarks
	230 $m\mu$	270 $m\mu$		230 $m\mu$	270 $m\mu$	
Halibut liver oil	17	—	Band 230 $m\mu$	59	55	Max. at 270, 230 $m\mu$
Turbot liver oil	20	—	Flat end abs.	120	160	Max. at 270 $m\mu$
Whale oil	6	—	End abs.	77	62	Max. at 230, 270 $m\mu$
Seal body oil	9.5	—	Band 230 $m\mu$	100	150	Max. at 270 $m\mu$
Roach body oil	20	—	Band at 230 $m\mu$	140	170	Max. at 270, 230 $m\mu$
Liquid cod liver oil acids (7 days' sap.)	—	—	—	180	280	Max. at 270, 230 $m\mu$

Table IV. *Individual acids*

Acid	Reputed i.v.	$E_{1\text{ cm.}}^{1\%}$ as obtained	Remarks	Time of refluxing hr.	$E_{1\text{ cm.}}^{1\%}$	Remarks
Lauric	0	—	—	32	0.9	—
Myristic	0	—	—	37	1.9	—
Palmitic	0	—	—	33	6.5*	End abs.
Erucic	75	—	—	37	8	End abs.
Oleic	90	13	Flat band 230 $m\mu$	23	16	Band 230 $m\mu$
Elaidic	90	12	End abs.	—	—	—

* This value is sufficiently high to suggest the presence of a small percentage of unsaturated impurity.

acids of palm kernel oil, red palm oil and olive oil, and was in no case increased more than some 50% by treatment with potash. It is noteworthy that olive oil consists mainly of the glyceride of oleic acid, and this evidence, supplemented by observations on a specimen of pure oleic acid, would appear to prove that the contribution of oleic acid to the increased absorption seen in the mixed acids of the more unsaturated fats is negligible. The absorption, after treatment with potash of the mixed acids of oils more unsaturated than olive oil, may be 10–70 times greater than the absorption before treatment. (Tung oil, which consists mainly of glyceride of elaeostearic acid, is exceptional.)

Although the vegetable acids afford the best indication of the nature of the acids which undergo spectroscopic changes the same conclusion may be deduced from the data in other groups. Thus the marine oils, which all contain considerable amounts of polyethylenic acids, also all show greatly increased absorption on treatment with potash. On the other hand, body acids of the fat deposits of mammals in several instances differ from acids from other sources in being present in the natural state largely in the absorptive form. We have no examples, among those examined, of highly unsaturated fats. In agreement with this the absorption after potash treatment is in no case very high. It may be noted that in the one bird fat examined (duck) the acids were present in the "pro-absorptive" state.

The absorption spectra shown after treatment with potash by the acids of various fats. Since the present work was carried out with a spectrophotometer giving only a low dispersion it was impossible to follow up the careful observations of Morton and his colleagues on the fine-structured absorption of the mixed acids, as shown notably by those of marine oils. Even with the condensed spectra available, however, the differences shown by marine oils on the one hand, and vegetable and terrestrial animal oils on the other, were quite marked. With vegetable oils, disregarding the notable exception of tung oil, the absorption maximum after potash treatment was always at about 230 $m\mu$. The same band was observed in the terrestrial animal acids. The absorption spectra of the

marine acids invariably showed a band at about $270\text{m}\mu$, and sometimes another at $230\text{m}\mu$, the relative intensities of absorption at these wave-lengths varying in different cases. Under the conditions of the present experiments the marine acids could readily be distinguished from all others by the development of the absorption maxima at $270\text{m}\mu$ after potash treatment. It must be emphasized, however, that the absorption developed by an arbitrary period of treatment with potash may be quite different from the maximum absorption attainable by the complete conversion of all the pro-absorptive acids present. Thus after very prolonged treatment with potash the mixed acids of linseed oil resemble those of the marine oils in showing intense absorption at about $270\text{m}\mu$, the absorption band in this case being due to the formation of solid unsaturated acids. (See following section.) Moreover the elaeostearic acids show intense absorption in this region even before treatment with potash. The acids absorbing at $270\text{m}\mu$ formed from marine oils appear to differ from those prepared from linseed oil and tung oil in being liquid, and in being formed more rapidly during potash treatment. Thus the liquid acids of cod liver oil, prepared by the separation of the solid acids by freezing, showed intense absorption at $270\text{m}\mu$ after 7 days' refluxing, but no sign of crystallization. This observation might possibly be explained by a greater solubility of a common solid acid in the liquid acids of cod liver oil, but it seems more probable that two separate acids, or groups of acids, both absorbing at about the same position, are involved.

II. THE FORMATION OF LIQUID AND SOLID "ABSORPTIVE" ACIDS FROM THE MIXED ACIDS OF LINSEED OIL

The data given in the preceding section permit a comparison of the amounts of "pro-absorptive" acids present in the mixed acids of various fats as measured by the rate of development of absorption. The procedure of refluxing for a fixed arbitrary period, however, will only give results of absolute significance in the case of those acids in which the whole of the potential absorption is developed within the time chosen. In the case of linseed oil more extensive experiments were undertaken in order to determine the time necessary for the changes to go to completion, and to examine the nature of the acids as finally present.

EXPERIMENTAL

Exp. 1. The mixed fatty acids of linseed oil were prepared by the method described above. A portion of the mixed acids was retained for spectroscopic examination and for the biological tests described in Section III. The remaining material was refluxed with a further supply of alcoholic potash, the top of the condenser being closed as soon as pressure equilibrium had been established in order to reduce risks of oxidation. Specimens were withdrawn at intervals and examined spectroscopically, with results shown in Table V.

The first effect of treatment with potash was a marked increase in absorption at $230\text{m}\mu$. Subsequently absorption at this position remained stationary, but a second distinct band was developed at about $270\text{m}\mu$. This band eventually became almost as intense as that at $230\text{m}\mu$.

Table V

Time of saponification	$E_{1\text{ cm.}}^{1\%}$ at	
	230 m μ	270 m μ
1 min.	2.5	—
23 hr.	165	—
47 "	253	90
92 "	305	197
136 "	360	223
164 "	360	293

Separation of the mixed "absorptive" acids into liquid and solid fractions. In addition to the spectroscopic changes treatment with potash produced obvious changes in the physical state of the mixed acids. Whereas the "fresh" acids were almost completely liquid at room temperature, the "treated" acids contained a matrix of white solid. By adding an equal volume of cold alcohol and filtering a yield of solid acids amounting to some 10% of the mixture was separated. After several crystallizations from alcohol white lustrous crystals, M.P. 75°, were obtained. A spectroscopic examination of the solid acids, and of the mixed liquid acids (which actually still contained a substantial amount of the solid acid), gave the following results:

	$E_{1\text{ cm.}}^{1\%}$ at	
	230 m μ	270 m μ
Solid acids	150	1000
Liquid acids	400	140

It is obvious, therefore, that the absorption band at 270 m μ is due to the formation of a solid acid, or mixture of acids, which has very high absorptive power.¹

The solid acid had i.v. (by Wijs's method, 12 hr.) = 187 (by the method of Rosenmund & Kuhnhenh [1923], 10 min.) = 144, mol. wt. (by titration) 270. A specimen of the acids prepared in a subsequent experiment (Exp. 2) was found by Dr Weiler, of Oxford, to contain C 76.82%, H 10.63% and to have mol. wt. 301 (by Rast's method). The analysed specimen had been recovered by two recrystallizations from alcohol from a specimen which had been allowed to stand for some time in air and was probably slightly impure through the presence of polymerides. If instead of basing our calculations on the mol. wt. of 301 we take the theoretical value of 278, which approximates to the value obtained by titration, the elementary analysis corresponds closely with that of a triethylenic acid, C₁₈H₃₀O₂.

Exp. 2. In this experiment 170 g. of the acids of linseed oil, from which the solid acids originally present had been separated by freezing, were refluxed for 25 days with potash. The apparatus was closed and the flask completely filled with the reaction mixture, so that the amount of air left in it was negligible.

As the result of this treatment the absorption of the mixed acids rose to intensities similar to those observed in the previous experiment ($E_{1\text{ cm.}}^{1\%}$ at 230 m μ = 410, at 270 m μ = 330). The yield of solid acid, in three crops totalling 15 g., was of the same order. The solid acid absorbed rather more strongly at 270 m μ ($E_{1\text{ cm.}}^{1\%}$ 1500) than the specimen obtained in Exp. 1. The increase may possibly be due to the fact that the original mixed acids had been freed from

¹ In comparison we may recall that $E_{1\text{ cm.}}^{1\%}$ at 328 m μ for vitamin A is about 1600, $E_{1\text{ cm.}}^{1\%}$ at 450 m μ for β -carotene about 2400.

solid constituents by freezing, and also to a more adequate number of recrystallizations. In this specimen the melting point also appeared to be slightly higher (77°). The band at $270\text{m}\mu$ was absent from the mother-liquor from which the solid acids had been crystallized ($E_{1\text{cm.}}^{1\%}$ at $230\text{m}\mu=425$, at $270=70$).

These results when compared with those in the preceding experiment indicate that the reaction in each case had gone to completion. This view was confirmed by an experiment in which the acids obtained in the mother-liquor were refluxed for a further week with potash. The absorption was little changed, with no evidence of an increase at $270\text{m}\mu$. In agreement with this there was no evidence of the formation of solid acids. (After additional treatment $E_{1\text{cm.}}^{1\%}$ at $230\text{m}\mu=445$, at $270\text{m}\mu=74$.)

The effect of prolonged treatment with potash on the acids of maize oil. Exp. 3. In order to throw light on the identity of the acids giving rise to solid isomerides a sample of the mixed acids of maize oil was refluxed for a week with potash. This oil is reputed to contain linoleic, but no linolenic or other polyethylenic acids. After treatment the usual band at $230\text{m}\mu$ was observed ($E_{1\text{cm.}}^{1\%}=110$) but there was no evidence of a maximum at $270\text{m}\mu$ ($E_{1\text{cm.}}^{1\%}<15$). No solid acids were formed.

The absorption of acids prepared from commercial boiled linseed oil. Exp. 4. In order to find whether absorption changes might be expected as the result of chemical treatment other than boiling with potash the mixed fatty acids of "boiled" linseed oil were examined. The specimen used was obtained through the kindness of Grindley and Co., and was described as a "lead and manganese boiled Calcutta linseed oil". The acids were prepared by a brief saponification, and were examined without further treatment. The intensity of absorption ($E_{1\text{cm.}}^{1\%}$ at $230\text{m}\mu=90$, at $270\text{m}\mu=9$) was considerably greater than for the untreated linseed acids, but much less than the maximum absorption obtainable by treatment with potash. In agreement with the absence of an absorption maximum at $270\text{m}\mu$ there was no sign of the presence of the solid unsaturated acids.

Comparison of the solid acid from linseed oil with the elaeostearic acids. The general character of the solid product obtained from linseed oil suggested that it might be identical with α -elaestearic acid of tung oil, or with the β -isomeride obtainable from the latter by distillation. According to Dingwall & Thompson [1934] these acids absorb strongly at $265\text{m}\mu$.

A specimen of tung oil was obtained through the kindness of Grindley and Co. Even before saponification this oil absorbed strongly at $270\text{m}\mu$, $E_{1\text{cm.}}^{1\%}$ being 1300. By a short saponification followed by several recrystallizations from alcohol of the solid acid so obtained, a specimen of α -elaestearic acid, M.P. 46° , was separated. This substance displayed the absorption typical of the oil, having $E_{1\text{cm.}}^{1\%}$ at $270\text{m}\mu=1800$. The I.V. was about 190. It was clear therefore that the two acids showed striking resemblances in both being solid, both highly unsaturated, and both having strong bands at $270\text{m}\mu$. The difference in melting points, however, ruled out their identity. A sample of α -elaestearic acid was next converted into the methyl ester, which was distilled under reduced pressure at 230° . The β -elaestearic acid obtained after the saponification of the distilled ester showed no change in its absorption ($E_{1\text{cm.}}^{1\%}$ at $270=1800$) at least as far as could be detected by the small spectrophotometer used in this work. The melting point of this isomeride (70°) was so close to that of the new acid (as prepared in Exp. 1) as to suggest that they might be the same substance in different degrees of purity. The melting point of the mixed materials was however depressed to 62° , demonstrating that they were not identical.

After storage (in bottles containing air in a refrigerator) a further difference in the acids became apparent. Under these conditions the tung acids formed a vitreous mass within a few weeks. The linseed acids, although eventually showing signs of deterioration, were more stable.

The behaviour towards bromine of the acids of linseed oil after refluxing with potash. *Exp. 5.* The acids of tung oil differ from those of linseed oil in their failure to form an ether-insoluble hexabromide. It appeared of interest therefore to examine the effect of treatment with potash on the amount of insoluble bromide formed by the acids of linseed oil. Portions of 3.6 g. each of the acids prepared by a short treatment with potash and by prolonged treatment (*Exp. 2*) respectively were dissolved in 40 ml. of dry ether. The solutions were cooled in a freezing mixture, and 2 ml. of bromine were added to each in small instalments over a period of 30 min. A distinct difference in the behaviour of the two solutions was apparent. With the acids prepared by a short saponification the usual heavy precipitate of crystalline "hexabromide" was formed. With the acids subjected to prolonged treatment with potash, however, there was no crystallization during the addition of bromine. The solution remained clear after standing in an ice-chest for some hours, and only a slight sediment appeared after keeping under similar conditions for some days.

The absorption spectra of the acids recovered from the ether-soluble and ether-insoluble bromides of the linseed acids. *Exp. 5a.* In the above experiment a yield of crystalline "hexabromide" corresponding to a theoretical linolenic acid content of 14% was obtained from the linseed acid prepared by a short saponification as a heavy white powder. This product, and also the syrupy liquid bromides, were next debrominated by refluxing with zinc and a methyl alcoholic solution of hydrochloric acid. The methyl esters so obtained were refluxed with potash for a week, and the free acids recovered and their absorption spectra determined. The results obtained were as follows.

Acids from	Short saponification	After 7 days' refluxing with KOH	
	$E_{1\text{ cm.}}^{1\%}$ 230 $m\mu$	$E_{1\text{ cm.}}^{1\%}$ 230 $m\mu$ 270 $m\mu$	
Crystalline fraction (14% of total)	5	670	610
Non-crystalline fraction (86% of total)	?	310	200

It will be seen that after refluxing the acids recovered from the "hexabromide" absorbed much more strongly than those recovered from the ether-soluble bromides. In neither case, however, was the absorption confined to one maximum, as might perhaps have been expected if only one type of pro-absorptive acid had been present in each fraction. The purity of the methyl "linolenate" which should in theory [Rollet, 1909] be the sole substance obtained by the debromination of the crystalline fraction, was also open to question on account of the lowness of the iodine value (i.v. before refluxing with KOH, Wijs's = 210, theoretical i.v. for methyl linolenate = 261). This low value, however, may conceivably have been caused by the incomplete removal of solvent.

Exp. 6. 36 g. of linseed oil acids prepared by a short saponification (*Exp. 2*) were dissolved in 360 ml. of dry ether and treated with slight excess (20 ml.) of bromine, which was added drop by drop over 30 min., the flask being cooled in a freezing mixture. The flask was allowed to stand for 2 hours in a refrigerator. The precipitated bromides were then filtered off, and washed many times with

small volumes of ether. 10.4 g. of white crystals were obtained, together with 5.1 g. of crude bromides which were recovered from the sides of the vessels. The total yield of crystalline bromides was thus 15.5 g., representing a theoretical linolenic acid content of 5.7 g., or about 16%. Excess of bromine was removed from the mother-liquor by treatment with zinc dust. The solution was then concentrated to a viscous liquid, which turned slightly brown during the course of the evaporation.

The crystalline bromides, a portion of the non-crystalline bromides and a portion of the original mixed acids (as a control) were then treated as in the previous experiment, except that refluxing was for 6 days. Iodine values before and after refluxing were determined by the method of Rosenmund & Kuhnhehn, the reagent being applied for 15 min. (Table VI).

Table VI

	i.v. short saponification m μ	After 6 days' refluxing with KOH		
		i.v.	$E_{1\text{ cm}}^{1\%}$	
			230 m μ	270 m μ
Original mixed acids	175, 173	—	—	—
Mixed acids treated with Zn + HCl	179	146	400	270
Acids from crystalline bromides, 16% of total	238, 247	204	490	420
Acids from non-crystalline bromides, 84% of total	152	128	300	190

The results of this experiment, while showing minor quantitative divergences, are substantially in agreement with those obtained in the preceding experiment.

DISCUSSION

The presence in linseed oil of two or more acids susceptible to the influence of treatment with potash. The above results suggest that linseed oil contains two acids, or two types of acids, which undergo changes as the result of treatment with potash. One acid gives rise to a product which remains liquid at room temperature, and which shows intense absorption at 230 m μ . The other acid becomes solid as the result of treatment with potash and has a very intense and distinctive absorption band at about 270 m μ . The correctness of this interpretation of the results, rather than of the alternative view that the absorption bands at 230 and 270 m μ refer to successive stages in the action of potash on one acid, seems clear from the observation that the absorptions shown after 1 week or after 4 weeks' treatment with potash were virtually identical. Moreover, after the solid acids have been removed, the absorption of the remaining liquid acids was unaffected by further treatment with potash.

Similarity of the solid linseed acid with the elaeostearic acid of tung oil. Although as pointed out above differences in melting-point rule out the identification of the solid acid obtained from the linseed acids by treatment with potash with either α - or β -elaostearic acid, its resemblance to these substances is so striking as to suggest that it must possess a closely similar constitution. Crystalline form in itself is a rarity in polyethylenic acids. The absorption in the ultraviolet region, both in regard to its position and its very high intensity, is also remarkable and very similar in each case. Finally we have seen that when the mixed acids of linseed oil are subjected to prolonged treatment with potash, so as to

cause the formation of the new solid acid they fail, in common with the tung acids, to form a crystalline ether-insoluble hexabromide. In view of the common possession of these striking characteristics it would appear reasonable to classify the new acid with the elaeostearic acids.

The mechanism underlying the spectroscopic changes. Before drawing any inferences as to the mechanism underlying the spectroscopic changes decisions must be reached on two points (1) whether oxidation takes place, and (2) whether the increase in spectroscopic absorption is related to a fall in iodine value. The finding that the changes may be effected under virtually anaerobic conditions presumably rules out the possibility of oxidation. On the other hand, the conclusion of Dann *et al.* [1935] that the spectroscopic changes are not associated with a fall in iodine value was based on experiments in which fats containing only small amounts of "pro-absorptive" acids were examined, or in which the time of treatment with potash was insufficient to carry the spectroscopic changes to completion. Under these circumstances slight falls in iodine value were usually observed, but were not considered to be significant. The results of the present work, however, taken in conjunction with the evidence of Edisbury *et al.* [1935] on fish liver acids, suggest that the spectroscopic changes are essentially related to a limited fall in iodine value. If this is so two alternative mechanisms appear to be possible: (1) we may assume, in agreement with Edisbury *et al.*, that the action of potash causes cyclization, or (2) we may postulate that isomeric changes take place which result in the failure of one or more of the unsaturated linkages to absorb the halogen through some form of steric obstruction.

Spectroscopic evidence for the formation of hydroaromatic polycyclic nuclei has been advanced by Edisbury *et al.* On the other hand, the close similarity between the solid acid obtained by the action of potash on the linseed acids and elaeostearic acid from tung oil recalls the work of Böseken [1929] on the constitution of the latter acid. Although from the iodine value the presence of only two unsaturated linkages would be suspected, experiments in catalytic hydrogenation suggested the existence of three such linkages. From this evidence, from the high exaltation of the refractive index and from a study of degradation products the presence of a system of three conjugated unsaturated linkages was postulated. If this evidence is accepted it would appear highly probable, from the spectroscopic similarity, that the solid acid obtained from linseed oil contains the same system of unsaturated linkages, the difference in melting-point being ascribable to some minor difference, such as the position of the unsaturated system in the carbon chain. Other possibilities, such as multiple isomeric changes of the *cis-trans* type, must however be borne in mind.

The identity of the "pro-absorptive" acids. The certain identification of the "pro-absorptive" acids which give rise on treatment with potash to acids showing absorption bands at 230 and 270m μ respectively is virtually impossible in view of the uncertainty of our knowledge as to the exact composition of the mixed acids of linseed oil. If we accept the current opinion that maize oil contains no acids more unsaturated than linoleic acid (two unsaturated linkages) then the absence of absorption at 270m μ from the mixed acids of this oil after refluxing with potash would suggest that linolenic (three unsaturated linkages) is the "pro-absorptive" acid responsible for the development of absorption in this position in the mixed acids of linseed oil. If we further accept the view that the solid acid from linseed oil is similar in constitution to the elaeostearic acid of tung oil, then the conclusion of Böseken that the latter acid contains three unsaturated linkages strongly supports the above hypothesis. On the other

hand, since the acids of maize oil develop absorption at $230\text{m}\mu$ on treatment with potash, it would appear reasonable to assume that the acid absorbing at this position is derived from linoleic acid.

The two attempts to confirm these conclusions by studying the effect of potash on the fractions obtained from the mixed acids by precipitation with bromine were however unsuccessful. In both experiments a concentration of potential absorption was observed in the solid bromides. This absorption, however, was not restricted to the $270\text{m}\mu$ position, absorption at $230\text{m}\mu$ being present in slightly greater intensity. Moreover, although there was a concentration of potential absorption in the crystalline fraction, the larger non-crystalline fraction (84 %) carried a much greater proportion of the total potential absorption at both positions. In agreement with the conclusion that the total amount of potential absorption was not influenced by treatment with bromine the absorption in the non-crystalline fraction, at both wave-lengths, was slightly depressed below the level of the original mixed acids.

Two alternative explanations of the disparity of the general evidence and that obtained in the fractionation experiments are possible. On the one hand we may accept the conclusion of Rollet [1909] that the crystalline bromide fraction represents pure linolenic acid. In this case we must assume that linolenic acid gives rise on treatment with potash to two products absorbing at 230 and $270\text{m}\mu$ respectively. Moreover, since the experiments with maize oil acids show that linoleic acid does not yield products absorbing at $270\text{m}\mu$, it is plain that the absorption at this position obtained by treatment with potash of the non-crystalline fraction must also be derived from linolenic acid. This finding would agree with Rollet's suggestion that only about one-fifth of the total linolenic acid in linseed oil is precipitated by bromine. Assuming that the linolenic acid in the non-crystalline fraction gave rise to absorption at 230 and $270\text{m}\mu$ in the same proportion as that of the crystalline fraction, the proportion of linolenic acid necessary to satisfy the spectroscopic data would be calculated as about 50 %. This percentage is in good agreement with the 50–60 % suggested by Rollet, but unless the remaining acids consisted entirely of oleic acid the iodine value calculated on this basis for the mixed liquid acids would be unduly high.

As an alternative to assuming the formation of two distinct absorptive acids from the one pro-absorptive acid, we may question the homogeneity of the acids derived from the crystalline fraction. It is perhaps sanguine to expect that bromine should precipitate pure linolenic acid as its hexabromide from a mixture of linolenic with other acids, including in all probability linoleic acid and possibly also small amounts of more highly unsaturated acids. From the spectroscopic data in the present work it might be expected that only about one third of the crystalline fraction consisted of linolenic acid. Some difficulty lies in the acceptance of the simple course of writing down the remaining two-thirds of the crystalline fraction as linoleic acid. In Exp. 4, and in the work of other investigators, the iodine value of the "crystalline" fraction was much greater than that ascribable to a mixture of one third of linolenic and two thirds of linoleic acid. Moreover, the absorption at $230\text{m}\mu$ was also increased in the crystalline fraction, whereas the linoleic acid concentration in this fraction must presumably have been somewhat less than in the "non-crystalline" fraction. These difficulties could be overcome by assuming the presence of an acid having four unsaturated linkages, and capable of giving rise on treatment with potash to an absorptive acid having more intense absorption at $230\text{m}\mu$ than that derived from linoleic acid. Thus one may visualize the action of

bromine as the precipitation of a fraction containing a concentration of the more highly unsaturated acids, i.e. having two, three and four unsaturated linkages, and with a mean degree of unsaturation slightly below that of linolenic acid. The non-crystalline fraction, on the other hand, would contain a concentration of less highly unsaturated acids, having one, two and three unsaturated linkages, with a mean iodine value slightly below that of linoleic acid. The most weighty argument against this view would appear to lie in the apparent purity of the linolenic acid hexabromide obtained by Rollet and other workers, as indicated by crystalline form and constancy of melting point.

III. THE BIOLOGICAL ACTIVITY OF THE ACIDS OF LINSEED OIL IN DIFFERENT SPECTROSCOPIC STATES

The work of Burr & Burr [1929] and of McAmis *et al.* [1929] has proved that rats are unable to survive when restricted for prolonged periods to diets deficient in fat. Experiments by Burr & Burr [1930] and by Burr *et al.* [1932] on the action of various fats and fatty acids in curing the lesions caused by fat deficiency have led to the identification of linoleic and linolenic acids as "essential fatty acids" (vitamin F) which presumably cannot be synthesized in adequate amounts by warm-blooded animals.

This work has been criticized by several groups of investigators who have either failed to confirm the necessity for the inclusion of fat in the diet, or have ascribed the lesions developed to an inadequate supply of the vitamin B complex. Burr & Brown [1933], in answering these criticisms, have pointed out that the lesions of "essential acid deficiency" are not to be expected unless the diet is rigorously freed from fat, and that they occur even when requirements for the vitamin B complex have been completely satisfied. The fact that some of the lesions observed in "essential acid deficiency" (notably scaliness of the skin and necrosis of the tail) also occur in deficiency of the vitamin B complex must be taken as evidence that these conditions are not specific for any single deficiency. Tunge [1932], Evans & Lepkovsky [1933] and Becker [1935] have confirmed the observations of Burr & Burr under the conditions specified by these workers.

Since the "essential fatty acids" are presumably to be included among those capable of showing changes in spectroscopic absorption, it was obviously of interest to inquire into the influence of the spectroscopic state on the biological activity.

EXPERIMENTAL

Basal diet. Albino rats, 4-6 weeks old, were given a diet of 25% caseinogen, 70% cane sugar, 5% salts and 10% of extracted dried yeast. The caseinogen was extracted with hot alcohol by the makers (Joseph Nathan, Ltd.). The dried yeast, in one large batch of 12.7 kg., enough to last the whole experiment, was finely ground and extracted twice with 20-litre portions of cold trichloroethylene. This treatment extracted 0.69% of lipoids. A subsequent extraction of a sample of the prepared yeast with chloroform gave a further 0.6% of lipoids, but since the biological tests had been started it was thought inadvisable to interfere with the continuity of the basal diet by resorting to further extractions.

During the first 156 days of the experiment liberal doses of vitamin A (500 γ per week of vitamin A concentrate, B.D.H.) and of vitamin D (1 γ per

week of calciferol) were given, the diluent being liquid paraffin. By a misunderstanding dosing was stopped after this point, but colorimetric determinations of vitamin A on the livers of four animals which died after being on experiment about 150 days indicated the presence of substantial reserves in all cases (mean total reserve 1000 I.U.). From the work of Davies & Moore [1937] reserves of this order should have been quite adequate for the subsequent period during which doses of fatty acids were given. Vitamin E was given from day 80 to day 295 as 40 mg. monthly of the unsaponifiable matter of cotton seed oil, partially freed from sterols. This concentrate was not tested biologically. No instances of the abnormality of the uterus found by Martin & Moore [1936] to occur invariably in rats given diets deficient in vitamin E were, however, observed.

With the growing complexity of our knowledge of the vitamins the technical difficulties in devising a diet free from the vitamin to be tested, yet containing adequate amounts of all other vitamins, are becoming increasingly serious. The above diet cannot be considered ideal since, apart from accidental imperfections, the factor found by Coward *et al.* [1929] in light white caseinogen was presumably absent. The results given below, however, indicate that the diet was adequate for the purpose required, since the survival of the animals was secured by the addition of the fatty acids under investigation. These acids could reasonably be presumed to be entirely free from any of the factors whose adequacy in the basal diet might appear open to question.

Behaviour of rats on the deficient diet. Weight increase curves of rats restricted to the above basal diet are shown in Fig. 1. In all cases growth ceased within 90–120 days, at body weights usually between 150 and 200 g. for males, and between 100 and 150 g. for females. In possible association with the more rapid growth rate of the males it is noteworthy that seven out of an original eleven males died before the commencement of dosing at 175 days, whereas all of the ten females were surviving at this time.

In the course of the tests instances of all the lesions reported as being typical of deficiency of “essential fatty acids” were observed. Scaliness of the tail, with occasional necrosis of the tip, was present in most cases. Apart from the tail the skin lesions developed were, however, usually less severe than those reported by Burr & Burr, possibly on account of the incomplete removal of lipoids from the dried yeast used in the basal diet. Generally they amounted to no more than a baldness and reddening of the tip of the nose, but a few cases of marked baldness or sparseness of the coat were noticed. In one animal, which had been cured by the administration of acids and then restricted once more to the basal diet (see below), a severe seborrhoea, with loosening of the hair in tufts, was developed. Two typical instances of the reddening of the eyelids described by McAmis *et al.* were observed. In one case, in which the eyeball was also involved, the lesion bore a striking resemblance to the xerophthalmia of vitamin A deficiency. A substantial vitamin A reserve was however detected in the liver at autopsy.

In order to confirm the occurrence of the kidney lesions reported by Burr & Burr, and subsequently studied histologically by Borland & Jackson [1931], the kidneys of most of the rats used in the present experiment were taken at autopsy and examined both macroscopically and in stained sections. In the majority of cases yellowish concretions (calcification) at the apices were observed. The tubules were usually much dilated, both in the cortex and medulla, while in some cases there was a marked degeneration of the epithelium of the convoluted tubules, or a blocking of the tubules with colloidal casts. These findings are essentially in agreement with the detailed description given by Borland & Jackson. The

greatly increased water intake of the animals during life, reported by Burr & Burr, was not systematically studied. In the few cases examined however the amounts of water ingested certainly appeared to be in excess of the requirements of normal animals.

Fig. 1. Biological tests on the mixed acids of linseed oil in different spectroscopic states. Rats Nos. 1, 2 and 3 were given 64 mg. daily of the mixed acids of linseed oil, in the "pro-absorptive" state at the points indicated. Nos. 3, 4 and 5 received similar doses of the acids in the "absorptive" state. Rats Nos. 7-21 were negative controls.

The administration of the mixed acids of linseed oil in different spectroscopic states. The mixed acids employed were those prepared in the course of Exp. 1, Part II. The specimens, prepared by a short saponification and by a week's refluxing with alcoholic potash respectively, were administered without esterification to two groups of three rats each at the rate of four drops (about 64 mg.) daily. Dosing was commenced on day 175 of the experiment, and continued until day 247. In every case a slow, but definite, increase in weight was observed over the period of dosing (see Fig. 1).

The effects of dosing with acids were also apparent in the subsequent history of the rats beyond the period covered by the weight curves. When dosing was started eight animals remained as negative controls. Two were killed for pathological examination. Of the remainder one animal (♀ No. 17) died during the period when the selected groups were still receiving acids, while four more (♂ No. 14 and ♀ Nos. 15, 16 and 19) died on or before the 303rd day of the experiment. At this period only one member of the untreated group (♀ No. 18) survived, without having shown any increase in weight. In contrast all six animals which had received acids survived at this point, although in most

instances their weight had again started to decline. Thus six out of six animals had survived in the treated groups, one out of six in the untreated group.

Administration of the solid unsaturated acid. Owing to scarcity of material it was only possible to test the biological activity of the solid acid on two animals. The tests were carried out on separate occasions, and in neither case were the technical conditions entirely satisfactory. Rat No. 18 ♀ was given 64 mg. daily of the solid acid (Exp. 2, Part II) for about 40 days, commencing on day 315 of the experiment. No weight increase was observed, but it is conceivable that the failure to respond was due to deficiency of vitamin A. For the other test rat No. 6 ♀ was used (see Fig. 2). This rat had been used for testing the activity of the mixed "absorptive" acids (Fig. 1) and subsequently allowed to decline again on the basal diet. Vitamin A had been supplied for several weeks without inducing any gain in weight. The solid acids were given at the level of 100 mg. daily for 10 days. No increase in weight was observed, and in spite of the administration of the acids death supervened 2 days after the conclusion of dosing. This test may possibly be open to the criticisms that the period of dosing was relatively short and that the condition of the animal may have reached an incurable stage.

The effect of the addition of 30% of linseed oil to the diet of rats after prolonged restriction to a diet deficient in essential fatty acids. In order to confirm the beneficial effects of the "essential fatty acids" the rats previously dosed with either "pro-absorptive" or "absorptive" acids (Nos. 1-6, Fig. 1) were allowed to decline again on the basal diet. The continuation of the weight curves, from the points of maximum weight after the administration of acids downwards, are shown in Fig. 2. At the points indicated (v.A) any possibility of vitamin A

Fig. 2. The treatment of rats allowed to decline for a second time through "essential fatty acid" deficiency with linseed oil. A vitamin A concentrate was given at the points marked "v. A". Linseed oil was included in the diet of rats Nos. 4, 2 and 1 at the level of 30 % from the points marked "L.S.O." Rat No. 6 was given 100 mg. daily of the solid unsaturated acid prepared from linseed oil at the point marked "S.A." Rats Nos. 5 and 3 were negative controls.

deficiency was ruled out by regular dosing with vitamin A concentrate, dissolved in liquid paraffin. No response was shown in at least four animals (Nos. 2, 1, 3 and 6). Slight irregular fluctuations occurred in the weight of ♀ No. 5. In the case of ♂ No. 4 growth appears to have recommenced from this point, but it is possible that the rise for the short period until treatment with linseed oil was

started may have been in reality no more than the commencement of a minor casual fluctuation in weight. At the points indicated by L.S.O. three of the animals were given linseed oil, which was included in the diet at the level of 30% in place of a corresponding amount of sugar. To avoid oxidative changes the diet was mixed fresh daily.

One animal (♂ No. 1) was by this time in a state of rapid decline and died 3 days later in spite of receiving linseed oil. In the other two rats prolonged periods of rapid growth were observed, which were accompanied by great improvement in the general condition of the animals. Thus the coats became sleek and glossy, with a restoration of the growth of hair on the nose and the healing of tail lesions. On autopsy at the conclusion of the experiment the viscera were found to be in a correspondingly healthy state, with abundant intraperitoneal fat deposition. Histological examination of the kidneys showed them to be normal, apart from a slight pitting of the cortex in one instance suggestive of healed lesions. The condition of the animals which were not given linseed oil remained poor.

DISCUSSION

The results of the above experiments afford additional confirmation of the findings of Burr & Burr, both in regard to the lesions developed in the rat when given a diet deficient in fat, and the cure of these lesions by the administration of small amounts of polyethylenic acids. Moreover, it would appear from the present work that the deficiency may be developed in fatal form using diets which have been less carefully freed from fats than those originally described by Burr & Burr.

The biological activity of the acids of linseed oil after prolonged treatment with potash. The specimen of mixed "absorptive" acids subjected to biological tests in the present work could not have contained more than traces of polyethylenic acids present in their original "pro-absorptive" form. The positive responses observed, therefore, must be ascribed to one or other of the "absorptive" acids, or to their combined action. As stated above the separate tests carried out on the solid acid, showing absorption at $270\text{m}\mu$, were somewhat inconclusive. The result given, however, might well have been anticipated from the finding of Burr & Burr that the elaeostearic acids, to which the new solid acid bears a striking resemblance, are devoid of biological activity. In support of this view no evidence of preformed absorption at $270\text{m}\mu$ was observed in any mammalian fat in the present work. On the other hand, in several fats (e.g. butter, lard and mutton fat) virtually the whole of the polyethylenic acid content appears to be present in the "absorptive" form, with a single absorption maximum at $230\text{m}\mu$. It would seem probable that the acid, or acids, absorbing at $230\text{m}\mu$ which are formed by treatment of the corresponding "pro-absorptive" acids with potash are identical with the acids absorbing at this position as found in mammalian fats.

The question of the purity of preparations of polyethylenic acids. Although the results of the present experiments completely confirm the observations of Burr & Burr it cannot, in the author's opinion, be taken as finally proved that linoleic and linolenic acids are themselves the "essential fatty acids". All that can be said is that the purest preparations of these acids so far available possess biological activity. No evidence as to the purity or otherwise of specimens of linoleic acid was obtained in the present work. The attempt to prepare a specimen of pure linolenic acid, however, resulted in a product which, on spectroscopic grounds, might be suspected of being a mixture of two or more

acids. The possibility that the biological activity of linoleic and linolenic acids may be ascribed to the presence of a small admixture of some more highly unsaturated acid, or acids, cannot be entirely dismissed.

SUMMARY

Part I

The effect of prolonged refluxing with potash in increasing the spectroscopic absorption of the mixed acids of various fats and oils has been studied. An arbitrary period of 24 hours' refluxing was adopted in most experiments. Under these conditions the absorption developed in the acids of vegetable oils, which were nearly transparent when prepared by a brief saponification, increased with the degree of unsaturation. The absorption maxima were at about $230\text{m}\mu$. The acids of land mammals' fats differed from those of vegetable fats in having a considerable portion, and sometimes virtually the whole, of their potential absorption developed in the natural state. The absorption maxima were again at about $230\text{m}\mu$. The acids of marine oils differed from those of the oils of vegetables and land mammals in developing absorption at about $270\text{m}\mu$, together with absorption at $230\text{m}\mu$ at slightly inferior intensity. The one bird fat examined had its acids present in the "pro-absorptive" form, with the development of a maximum at $230\text{m}\mu$ on refluxing.

The acids responsible for the increased absorption would appear to be those having two or more unsaturated linkages. Saturated acids and oleic acid do not show the property in any marked degree.

The absorption properties of the acids of tung oil are exceptional since this oil shows very intense absorption, with a sharp maximum at about $270\text{m}\mu$, even before saponification.

Part II

A detailed study of the effect of refluxing with potash on the mixed acids of linseed oil has been carried out with a view to determine the time necessary for the changes to go to completion and to investigate the nature of the products formed. The first effect of refluxing was to raise the absorption at $230\text{m}\mu$. Later, however, absorption at $270\text{m}\mu$ began to develop, almost equalling the absorption at $230\text{m}\mu$ in intensity after about a week. The development of absorption at $270\text{m}\mu$ was found to coincide with the formation of a solid unsaturated acid which could readily be separated by crystallization. The new acid bore a striking resemblance to the elaeostearic acids which may be prepared from tung oil, but had a higher melting-point than either the α - or β -isomeride. The mother-liquor of the mixed acids from which the solid acids had been crystallized showed absorption only at $230\text{m}\mu$, which was not appreciably changed by further refluxing.

The current view that linolenic acid and the elaeostearic acids are isomeric, considered in conjunction with the finding that absorption at $270\text{m}\mu$ was not developed by the mixed acids of maize oil, which is considered to contain no linolenic acid, suggested that the new solid acid might be derived from linolenic acid. "Crystalline hexabromide" and "non-crystalline bromide" fractions were prepared from the mixed acids of linseed oil. A slight concentration of potential absorption was found in the "crystalline" fraction, which should presumably have consisted of almost pure linolenic acid, but absorption was developed both at 230 and $270\text{m}\mu$. The bulk of the potential absorption at both these positions remained in the much larger "non-crystalline fraction". These results suggest either that the acid may give rise on treatment with potash to separate products

absorbing in different positions, or that the "hexabromide" fraction was in reality a mixture.

In common with the acids of tung oil the mixed acids of linseed oil, after prolonged refluxing with potash, no longer gave a crystalline ether-insoluble precipitate with bromine.

A commercial sample of boiled linseed oil gave acids showing substantially increased absorption at $230\text{m}\mu$, but no absorption was present at $270\text{m}\mu$.

Part III

The results of Burr & Burr on the inability of the rat to subsist on a diet freed from fat, and on the nature of the lesions sustained, have been confirmed. The mixed acids of linseed oil, both in the "pro-absorptive" state as prepared by a brief saponification and in the "absorptive" state after prolonged treatment with potash, were found to have biological activity in curing the condition. It is doubtful whether the solid acid absorbing at $270\text{m}\mu$ has biological activity.

The only possible criticism of the conclusion of Burr & Burr would appear to lie in the doubtful purity of even the best preparations of linoleic and linolenic acids, which may possibly contain small amounts of more highly unsaturated acids.

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