

A Contribution to the Discussion of the Glyceride Structure of Stearic-Rich Animal Depot Fats

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CONSIDERABLE attention has been given in recent years in several laboratories in the United States to the typical glyceride constitution—often marked by relatively high proportions of trisaturated glycerides in relation to the ratio of saturated to unsaturated acids in the whole fat—of animal body fats such as those of sheep and oxen. The experimental facts are, apart possibly for minor details, not a matter of dispute, but differing interpretations have been given to them, the discussion having centered largely on whether the fats of this category (rich in stearic glycerides) follow the “rule” of “even” or of “random distribution.” Possibly, to a certain extent, there has developed at times some little confusion as to what exactly is meant by “even” or by “random” distribution of fatty acids amongst glycerol molecules, leading in any case largely to the assemblage of complex mixtures of mixed triglycerides in natural fats. It is hoped in the course of this communication to clarify uncertainties which may have arisen on this score and also to define without ambiguity the views upon the specific and characteristic glyceride structures of stearic-rich animal body fats which have been developed and maintained by the author and his colleagues at the University of Liverpool over a period of some 15 years.

From consideration of the results of studies in some detail of the component glycerides of a number of ox, sheep, and pig body fats in the Liverpool laboratories at different times (1, 2, 3, 4, 5) it was concluded that the typical structure of stearic-rich animal depot fats is best explained by the hypothesis first advanced by Banks and Hilditch (6), namely, that the more saturated (stearo-) glycerides have been produced by hydrogenation of a mixture of preformed oleo-glycerides (notably palmitodioleins), the latter having been built up in accordance with the principle of “even” or “widest” distribution (7) of each fatty acid amongst the glycerol molecules. Longenecker (8), on the other hand, has maintained that in animal fats, in contradistinction to vegetable fats, the glycerides are assembled on a “random” basis more or less in accordance with calculations based upon the law of probability. Norris and Mattil (9) have recently supported this alternative view and have not failed to remark that it involves implicitly a fundamental difference in the synthetic action of the plant and animal lipolytic enzymes concerned.

The phenomenon of “even” distribution of fatty acids in the glycerides of natural fats, as understood in our Liverpool school, has recently (7) been outlined in the following terms (which are perhaps preferable to attempting a rigid definition in a single sentence):

“(i) When a given fatty acid A forms about 35% (mol.) or more of the total fatty acids (A+X) in a fat, it will occur at least once, G(A₂X), in practically all the triglyceride molecules of the fat in question.

“(ii) If it forms from about 35 to about 65% (mol.) of the total fatty acids (A+X), it will occur

twice, G(A₂X), in any given triglyceride molecule in some instances, and of course more frequently the higher the proportion of this acid in the total fatty acids.

“(iii) If it forms 70% or more of the total fatty acids, the remaining fatty acids (X) can at most only form mixed glycerides G(A₂X), and the excess of A then, and broadly speaking then only, appears as a simple triglyceride, G(A₃).

“(iv) A minor component acid which forms much less than about a third of the total fatty acids (e.g. 15% or less), will not occur more than once in any triglyceride molecule (and, of course, not at all in many of the triglyceride molecules).

“The above statement covers broadly the mode in which “even distribution” of fatty acids in glyceride molecules operates in the natural fats. In the majority of cases the effect is quite other than that which would result if the fatty acid molecules were distributed on an indiscriminate or random basis. In the case of completely random distribution the amount of a simple triglyceride G(A₃) which should be present in a fat is calculable and proportional to the cube of the percentage of the fatty acid A in the total fatty acids of the fat.”

Are animal depot fat glycerides constituted on a “random” (probability) basis? It is desired in the first place to examine, in the light of the earlier work (1. 2. 3. 4) from this laboratory and of the results given in the preceding paper (10), the suggestions of Longenecker (8) and of Norris and Mattil (9) that animal body fats approximate towards a “random” (probability) glyceride structure. These workers appear to have confined attention almost exclusively to the tendency towards coincidence between the observed content of *trisaturated glycerides* and the amount of the latter to be expected if it were proportional to the cube of the concentration of the saturated acids present in the total acids of the fat. At one time this was the only analytical comparison available, but the application of more complete preliminary resolution of a fat by the improved crystallization techniques gives also an adequate (if perhaps not rigidly quantitative) measure of the content of *triunsaturated glycerides* and therefore also of the two categories of mixed saturated-unsaturated glycerides. Hence it is now possible to compare (Table I) the observed values for each of the four categories of glycerides in seven animal body fats studied in this laboratory with the values calculated from the expression $S^3 + 3S^2U + 3SU^2 + U^3$ (where *S* and *U* are respectively the percentage proportions of saturated and unsaturated acids in the whole fats).

Table I illustrates well the general similarity between the observed and the calculated “probability” figures for trisaturated and for mono-unsaturated-disaturated glycerides; in the trisaturated glycerides there is nevertheless definitely greater proportional divergence between the observed and calculated values in the more unsaturated fats in the upper part

TABLE I
Observed and Calculated (Probability) Contents of Glyceride Categories in Animal Body Fats

		Component acids % (mol.)		Component glycerides % (mol.)				
		S	U	S	3	2	1	—
				U	—	1	2	3
(2) Pig (back).....	{ Found { Calc.	44.1	55.9	5 8.5	32 33	60 41	3 17.5	
(4) Sheep (back).....	{ Found { Calc.	50.2	49.8	5 13	42 38	53 37	0 12	
(2) Pig (perinephric).....	{ Found { Calc.	50.5	49.5	9 13	43 38	45 37	3 12	
(4) Sheep (perinephric).....	{ Found { Calc.	57.2	42.8	14 19	48 42	38 31	0 8	
(1) Ox (English).....	{ Found { Calc.	58.7	41.3	17 20	49 43	34 30	0 7	
(10) Sheep (Indian).....	{ Found { Calc.	60.6	39.4	28 22	28.5 43	40.5 29	3 6	
(3) Cow (Indian).....	{ Found { Calc.	67.5	32.5	28 31	51 44.5	20 21	0 3.5	

of the table than in the fats with a greater amount of saturated acids in the total fatty acids.

When the two other categories—diunsaturated and triunsaturated—of the animal body fat glycerides are similarly studied, it is however equally evident that (except in the most saturated instances) there is no accordance whatever between the found and the calculated values. In the diunsaturated monosaturated group the observed values are generally in large excess of those demanded by probability considerations: the observed figures for the two least saturated fats are nearly 50% greater than the calculated amounts. Similarly, and perhaps most significantly, only minor or negligible amounts of triunsaturated glycerides have been found in any of this series of fats; whereas from probability considerations there should have been quite appreciable triunsaturated glyceride contents in the less saturated specimens (12-17.5%). Equal divergence is seen in a lard examined by Riemenschneider *et al.* (11), in which the presence of a substantial quantity (17.6%) of triunsaturated glycerides was recorded. This fat, however, in addition to oleic acid (49.6%) contained 12.8% of linoleic and 1.2% of other polyethenoid acids—a total unsaturated acid content of 63.6%; the amount of triunsaturated glycerides deduced from probability considerations is therefore about 25%—approximately half as much again as that actually found.

The view that in animal depot fats the glycerides are constituted in a fashion which approximates to that arithmetically derived from the law of probability is thus seen to break down completely in so far as two of the four general categories of mixed glycerides are concerned—the triunsaturated and diunsaturated classes. Consequently the theory that “random” or “probability” distribution operates in animal fats although not in vegetable fats cannot be accepted as a satisfactory explanation. Moreover, whereas Norris and Mattil (*loc. cit.*) note the corollary that there must on this hypothesis be a fundamental difference in the synthetic action of plant and animal enzymes and add that the basic reason for such a fundamental difference would be well worth investigating, one would suggest rather that it is desirable first to consider *whether* so unlikely a divergence in fact exists or needs to be presumed.

The observed facts have indeed for long appeared to be susceptible of an explanation which does not

involve this corollary and which it is desired to take this opportunity briefly to recapitulate here. Before doing so, the evidence illustrated by the numerical data in Table I may be summed up as follows: There is no evidence whatever of “probability” considerations operating in so far as the proportions of triunsaturated and diunsaturated glycerides are concerned; on the other hand, there is distinct evidence of considerable approximation to this type of structure in the monounsaturated and trisaturated types of glycerides, and this becomes progressively more marked the greater the proportion of saturated acids in the whole fats.

Glycerides of animal body fats viewed as a special or modified case of “even” distribution. Norris and Mattil (*loc. cit.*) remark “The rather qualitative nature of Hilditch’s ‘rule of even distribution’ has hampered a reliable evaluation of its validity.” It seems therefore necessary to point out that it would be very remarkable if a mathematical formula could be reached which conformed accurately with the results of a sequence of natural operations leading to so complex a mixture as a natural fat. The most that can be expected is to reach generalizations which cover the general trend of the observed facts more or less adequately, and in this sense any explanation is bound to be rather qualitative in that it is not to be expected to afford a rigidly quantitative picture. As a matter of fact, chemists concerned with fats may find legitimate satisfaction that no other group of complex natural organic products is yet so susceptible to quantitative generalization even when it is admitted that the discussion of natural fats has been for the most part on broad and general lines.

For example, no vegetable fat has yet been observed to follow the “even distribution rule” with completely quantitative adherence. Two simple illustrations of very close, yet not wholly quantitative, adherence to the “rule” may serve to illustrate this point. The seed fats of *Garcinia indica* (Kokum butter) (12) and of *Theobroma cacao* (cacao butter) (13) both contain approximately 60% of saturated acids in their total fatty acids; trisaturated glycerides should be absent from each according to “even” distribution, but should amount to nearly 22% of the fats on probability considerations. The amounts of trisaturated glycerides actually observed were respectively 1.5% and 2.5%—definite but very small amounts indicating very close, but not absolutely

quantitative, adherence to the "even distribution rule."

Returning to the group of animal body fats, it may be recalled that in the early studies of glyceride structure at Liverpool (1927-30) the characteristic differences in trisaturated glyceride content between animal body fats and seed fats were observed (13, 14) and the resemblance of the proportions of animal fat trisaturated glycerides to those determined from probability considerations and to those in mixed glycerides synthesized from glycerol and fatty acids at 140-150°C. in a vacuum was pointed out by Bhattacharya and Hilditch (15). Thereafter, however, a number of rapidly accumulating data for different animal body fats caused Banks and Hilditch (6) to put forward in 1931-2 the tentative suggestion that the high trisaturated glyceride contents might be due to hydrogenation of a mixture of oleo-glycerides which had been synthesized by the animal on lines approximating to "even" distribution. Subsequent studies at Liverpool have uniformly supported and developed this hypothesis, the evidence in favor of which may be summed up as follows:

1. When the trisaturated glyceride contents of animal depot (and milk) fats are plotted against the proportions of saturated acids in the total fatty acids (16), they lie on a band which, when extrapolated to zero trisaturated glycerides, intersects the horizontal axis at about 30% of saturated acids, and not at the origin (as should be the case if probability considerations apply).

2. The content of palmitic acid in animal body fats has been shown (6, 17) to lie within the limits of $30 \pm 3\%$. Achaya and Banerjee (18) have recently shown that Indian animal fats contain 3-4 units per cent more palmitic acid than European animal fats and that the corresponding curve for Indian animal fats intersects the horizontal axis at about 35% of saturated acids. It follows that an animal fat containing little or no stearic acid has negligible proportions of trisaturated glycerides.

3. It also follows that the increase in trisaturated glyceride content occurs *pari passu* with increasing proportions of stearic acid in the total fatty acids while (with approximate constancy of palmitic acid content) oleic acid must diminish as stearic acid augments.

4. If, in the series of body fats discussed in Table I, the changes in the relative proportions of palmitodioleins, oleo-palmitostearins, and palmitodistearins are considered (Table II), it will be seen that the dioleoglycerides fall steadily as the amount of ste-

aric acid increases, the mono-oleoglycerides increase equally steadily, and the percentage of palmitodistearins rises from a very low figure to nearly 20%. (In Table II oleic and the minor amounts of polyethenoid C_{18} acids are included in the term "oleo"-glycerides.)

5. It has been shown (5) that progressive catalytic laboratory hydrogenation of a relatively unsaturated pig back fat causes increase in trisaturated glycerides to a corresponding extent to that observed for animal body fats of similar total saturated acid contents (16).

Saturation of some of the ethenoid bonds in a preformed mixture of oleo-glycerides by a bio-hydrogenation process may be visualized as a process of indiscriminate attack on double bonds which may undergo or escape addition of hydrogen, thus superimposing a "random" introduction of stearic radicals upon an originally "evenly distributed" pattern of oleic and palmitic groups. On this view, the greater the extent of such hydrogenation, the more will the content of trisaturated (and also monounsaturated disaturated) glycerides conform to that calculated from probability considerations. The data in Table I are in complete accordance with this concept, which, it is submitted, continues to offer the most consistent and logical explanation of the characteristic glyceride structure of stearic-rich animal depot fats.

Hilditch and Meara (19) have shown that, in fats which largely follow the "rule of even distribution," the chief component glycerides can be "computed" with some approach to accuracy from the percentage composition of the total fatty acids by arithmetically proportioning oleic (or other major component unsaturated) acid amongst the saturated acids and further combining these to give binary mixtures of oleodipalmitin and palmitodiolein, oleodistearin and steardiolein, etc. This "computation" naturally fails when applied to stearic-rich animal depot fats as a whole; but when the acids present as trisaturated glycerides are deducted from the total saturated acids, and the oleic acid is then partitioned between the remaining palmitic and stearic acids, accordance (often remarkably close) between the computed and the observed values for palmitodiolein, oleopalmitostearin, and the other main types of mixed oleo-saturated glycerides results. This may appear to be an additional argument in favor of the hypothesis given above.

Circumstances in which glyceride structure may appear to follow "random" distribution. In the light of the above discussion, the approximation to

TABLE II
Relative Proportions of Palmitodi-"oleins," "Oleo"-palmitostearins and Palmitodistearins in Animal Body Fats

	Pig Back (2)	Sheep Back (4)	Pig Perinephric (2)	Ox English (1)	Sheep Perinephric (4)	Sheep Indian (10)	Cow Indian (3)
C_{18} fatty acids:							
Stearic.....	13.8	14.5	17.6	21.4	25.6	26.8	27.9
Oleic + octadecadienoic.....	51.1	47.9	45.9	38.7	40.4	35.1	30.5
Palmitodi- C_{18} glycerides (Increments %):							
Palmitodi-"oleins".....	53	46	40	23	25	26	17
"Oleo"-palmitostearins.....	27	28	34	32	41	27	38
Palmitodistearins.....	2	2	5	6	10	11	12
	82	76	79	61	76	64	67
Palmito- C_{18} glycerides (%):							
Palmitodi-"oleins".....	65	61	51	38	33	40	25
"Oleo"-palmitostearins.....	33	37	43	52	54	43	57
Palmitodistearins.....	2	2	6	10	13	17	18

"random" or probability values for the trisaturated and monounsaturated glyceride groups of the more saturated animal body fats is in a sense accidental, rather than an indication that probability considerations determine the glyceride structure of these fats. It is well to point out that other circumstances can equally lead to a state of affairs in which the component glycerides of a fat, while conforming closely with "even distribution," may at the same time have the appearance of conforming equally with "random" distribution as calculated from probability considerations. In other words, resemblance to mixtures of glycerides calculated on probability considerations is often an accidental or numerical consequence which cannot properly be correlated with any clearly defined generalization as regards glyceride structure. The proportions of trisaturated glycerides in stearic-rich animal body fats are one illustration of this statement; two other instances may also be given:

1. When the proportion of saturated (or of unsaturated) acids in the total acids of a fat is comparatively high, the calculated values, on either an "even distribution" or a probability basis, for trisaturated (or triunsaturated) glycerides become increasingly close so that the experimental error involved in their determination does not permit discrimination between the alternative modes of combination. This is seen to some extent in Table I, (a) in the trisaturated glycerides of the pig back fat and (b) in the triunsaturated glycerides of the Indian sheep and cow fats. It obtains still more in seed fats with very high contents (over 80-85%) of saturated acids (20), such as the nut fats of the *Palmae*, e.g., babassu fat [cf. Jackson and Longenecker (21)].

2. The operation of "even" distribution is most readily perceived when the number of major component acids in a fat does not exceed three or at most four. If a fat contains a much larger number of acids (e.g., six or seven), all in proportions of the same order, the operation of the principle of even distribution leads to a result which is indistinguishable from that deduced from probability considerations. Thus marine animal oils contain a number of acids—palmitic, hexadecenoic, oleic, eicosenoic, eicosatetraenoic, eicosapentaenoic, docosapentaenoic, docosahexaenoic—in proportions of more or less the same order of magnitude, and Hilditch and Maddison (22) have recently shown that, although the constituent glycerides of whale, herring, and seal oils are mixtures of mixed glycerides assembled as would be expected from the operation of "even" or "widest" distribution, their proportions of trisaturated, mono-, di-, and triunsaturated glycerides at the same time accord fairly closely with the values calculated from probability considerations.

There is thus more than one way in which a fat, essentially "evenly" distributed in type, can nevertheless exhibit the appearance of "random" distribution.

The glyceride structure of fats synthetically prepared at elevated temperatures by esterification of glycerol with fatty acids. Bhattacharya and Hilditch (15) prepared synthetic triglycerides by heating with glycerol at 145° in a vacuum, in presence of traces of an aromatic sulphonic acid, various mixtures of saturated (lauric, palmitic, or stearic) and oleic acids. Plotting the content of trisaturated glycerides in the products against fatty acid composition they

obtained curves very similar to, but not quite identical, with that expressing the relationship

$$\% \text{ trisaturated glyceride} \propto (\% \text{ saturated acid})^3.$$

The experimental curves and the latter graph intersected in each case at the point corresponding with equimolecular amounts of saturated and unsaturated acids; when saturated acids were in excess of the oleic acid the observed percentages of trisaturated glycerides were slightly lower than those calculated by the probability equation, and conversely. These results showed that to a large extent the course of esterification of glycerol with a mixture of fatty acids at 145°C. followed the probability or "random" distribution rule.

Norris and Mattil (9) showed that an "evenly-distributed" mixture of mixed glycerides, such as those of soya bean oil or of cottonseed oil, or a mixture of tripalmitin and triolein, or a mixture of lard and hydrogenated lard, when heated with small proportions of stannous hydroxide catalyst at 225°C. for a short time, suffered interesterification and rearrangement of the constituent glycerides in such a way that the product contained trisaturated glycerides approximating to the amount indicated by probability considerations. Since direct esterification experiments had established the production of a "random" type of mixed glycerides at the elevated temperatures employed, it would be expected that the effect of a catalyst which promotes acyl interchange in glycerides at an elevated temperature would be to produce the "random" type which is evidently the stable form at these temperatures. Subsequently Naudet and Desnuelle (23) have described similar experiments in which oleodistearin and stearodiolein, or tristearin and triolein, were isomerized under similar conditions to those employed by Norris and Mattil; the product from an equimolecular mixture of tristearin and triolein was found to contain 11.3% tristearin, 38.6% oleodistearin, 39.2% stearodiolein, and 10.9% triolein [compared with the calculated (probability) values of 12.5% for tristearin and for triolein, and 37.5% for each of the mixed oleostearins].

It has thus been demonstrated by these various researches that the mixed glycerides produced at high temperatures either by esterification of a mixture of fatty acids with glycerol, or by acyl interchange (interesterification) of mixtures of selected triglycerides, form mixtures closely resembling those to be expected from the theory of probability. These results, interesting and valuable in themselves, do not seem however to have any bearing upon the problem of the specific structure of mixed glycerides elaborated by enzyme action in the living cell at (or only slightly above) the atmospheric temperature, and one is unable to accept the statement of Norris and Mattil that "a new approach to the glyceride structure of natural fats has been achieved through the application of enforced interesterification to samples of animal and vegetable fats." There will be general agreement with the further statement of these authors that the enzyme process exerts a selective action in the synthesis, with the consequence that (as so often happens) the products of the natural process are entirely differently constituted from those produced in the laboratory by high-temperature processes. But it is illogical and unnecessary to argue that the processes of lipolytic enzyme assemblage of fatty acids in

glycerides should differ in any fundamental manner in plants and in animals. On the contrary, it is maintained that the characteristic glyceride structure of stearic-rich animal body fats can be more satisfactorily accounted for by the alternative explanation which has been discussed in this communication and which has been accepted by the writer and his co-workers for some considerable time.

Summary

Comparative study of the constituent glycerides of a number of ox, sheep, and pig body fats which have been investigated in the Liverpool laboratories reveals that, while there is considerable accord between the values observed for the trisaturated and monounsaturated groups of glycerides with those calculated from probability considerations, there is complete divergence between the latter and the values observed for the di- and triunsaturated glycerides.

The views on animal body fat glyceride structure developed and maintained by Hilditch and co-workers are shown to be in harmony with the arithmetical relationships referred to (Table I), while they do not require the postulate that enzyme synthesis of glycerides should differ fundamentally in animal and in vegetable cells.

It is shown that apparent resemblance in the proportions of the four categories of saturated and unsaturated glycerides to those calculated on probability considerations may arise in a number of different ways, and that such resemblance has not necessarily any bearing on the mode of glyceride structure observed in fats.

The well-established fact that mixed glycerides produced at high temperatures by synthesis or by

acyl interchange approximate to "random" (probability) distribution also has no bearing upon the matter of the structure of mixed glycerides produced by the synthetic action of enzymes at or near atmospheric temperatures in the living cell.

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Report of the Oil Color Committee—1948

AT the Color Committee meeting held in New Orleans at the time of the Spring A.O.C.S. Convention it was the unanimous decision of the members to investigate during the coming year the reproducibility of the Coleman Jr. spectrophotometers and to attempt to determine just what readings would be most advantageous if a spectrophotometric method for color grading was set up. In order to accomplish this purpose 12 oils were submitted to 25 collaborators, each of whom was asked to read spectrophotometric transmissions in "B" type 25-mm. cuvettes at specified wavelengths, to read the Lovibond color, and to assign an arbitrary eye grade to the oils, assuming the lightest oil to have a grade of one and the darkest 25. Each collaborator was also asked to prepare and read the spectrophotometric curve on a solution of nickel sulfate prepared by dissolving 20.000 grams of $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ in water and diluted to a total volume of 100 ml.

Work Done

Twenty laboratories reported data on the oils submitted for examination. The complete spectral characteristics of the 12 oils are shown in Figures 1, 2, and 3. Eleven laboratories reported results on the nickel sulfate solution using the Coleman Jr. spec-

trophotometer while three laboratories reported using the Beckman spectrophotometer. The Beckman results are calculated to the measured diameter of the cuvettes used in the Coleman results. Figure 4 shows the composite data obtained on the Coleman Jr. and Beckman instruments for the nickel sulfate solution.

Discussion of Data

In discussing the data reported, it seems logical to divide the material into two parts, the first part dealing with the results obtained on the oil samples and the second part dealing with the results obtained on the nickel sulfate solution.

Data on Oil Samples

The composite data or the average data for all of the laboratories of the 12 oils which were submitted for transmission and other measurements is shown in Table I. One of the primary objectives of this work was to attempt to correlate density measurements at 550 $\mu\mu$, Lovibond colors, color index at more than one wavelength, and such other measurements as might seem desirable with eye gradings. At the bottom of Table I are shown the essential figures necessary for these correlations.