The Proportions of Different Lecithins in the Livers of Rats Deficient in Essential Fatty Acids

By F. D. COLLINS

*Russell Grimwade School of Biochemistry, University of Melbourne,
Parkville, N.2, Victoria, Australia*

(Received 3 September 1965)

1. Lecithin was prepared from the livers of rats deficient in essential fatty acids and analysed by means of countercurrent distribution. Thin-layer chromatography showed that only lecithin was present. 2. The distributions of phosphorus and the fatty acids at the 3 and 2 positions were determined. 3. It has been shown that 26% of the fatty acids in the 3 position were unsaturated and that most of the $\Delta^{5,8,11}$ -eicosatrienoic acid and the arachidonic acids occur as the stearyl or oleoyl lecithins.

Collins (1963) has shown that countercurrent distribution of lecithins gives a partial separation of stearyl from palmitoyl components, and applied this technique to a sample of rat-liver lecithins in which these two saturated fatty acids constituted half of the total fatty acids and could be assumed to be present in the 3 position. In this case the proportions of the various lecithins were calculated by a process of curve fitting. When the lecithins from the livers of rats deficient in essential fatty acids were examined the distribution of fatty acids was not as simple and the procedure has been modified so that the fatty acids at the 3 and 2 positions could be separately determined.

EXPERIMENTAL

Materials

The rats were an inbred strain of Sprague-Dawley obtained from Holtzman's Rat Farm, Madison, Wis., U.S.A. Male animals at weaning were put on a fat-free diet; they stopped growing after 15 weeks and showed the characteristic skin lesions of the deficiency. The diet contained 73% sucrose, 21% fat-free casein (British Drug Houses Ltd.), 4% salt mixture (U.S.P. XIV, 1950), 2% cellulose and 0.5% vitamin mixture (Nutritional Biochemicals Corp.). Radioactive phosphate was injected intraperitoneally as 0.45 mc of $H_3^{32}PO_4$ /rat, in 0.2 ml. of 1 mM-phosphate buffer, pH 7.4, containing NaCl (0.1 M).

All solvents were redistilled before use. DEAE-cellulose was obtained from Bio-Rad Laboratories, Calif., U.S.A. *n*-Propyl gallate was laboratory-reagent grade. Diazo-methane was prepared as described by Collins & Wheeldon (1957). Black-snake venom (phospholipase A, phosphatide acyl-hydrolase; EC 3.1.1.4), was obtained from Miss H. Doery, Commonwealth Serum Laboratory, Melbourne, Australia. The venom was dissolved in 0.3 M-ammonium acetate containing 12 mM- $CaCl_2$ so that 0.10 ml. contained 80 mg. of venom (Doery & Pearson, 1961).

Methods

Lipids were extracted and brought into reaction with 1-fluoro-2,4-dinitrobenzene as described by Wheeldon & Collins (1957). Lipid phosphorus was determined as described by Collins (1959). The purity of the isolated lecithins was tested by using thin-layer chromatography on silica gel G with chloroform-methanol-water (80:30:3, by vol.) as the solvent. Controls showed that lecithin, sphingomyelin and lysolecithin were well separated and, in addition, the presence or absence of free fatty acids and of methyl esters of fatty acids could be shown. Measurements of radioactivity were carried out as described by Collins (1963). The separation of the lecithin-sphingomyelin fraction was carried out in two steps by using the dinitro-phenylated lipids. The neutral fat was separated from the polar lipids by the method devised by Galanos & Kapoulas (1962) and the neutral phospholipids were separated from the acidic phospholipids by chromatography on DEAE-cellulose as described by Collins (1963).

Countercurrent distribution. This was carried out in an all-glass machine capable of 120 transfers and with a capacity of 20 ml. in each phase. The solvent system used was carbon tetrachloride-methanol-water (62:35:3.15, by vol.) containing 10 mg. of *n*-propyl gallate/l. as an anti-oxidant (Collins, 1963). The lecithin-sphingomyelin was added to the machine and 170 transfers were carried out in which 50 fractions were removed at the end of the train of tubes. This step eliminated most of the sphingomyelin and any traces of lysolecithin; after this the countercurrent distribution machine was reconnected to allow recycling and 160 more transfers were carried out. At this stage all the tubes which did not contain lecithin and some which contained sphingomyelin with a trace of lecithin were removed and replaced with fresh solvent and 70 more transfers carried out. Altogether 400 transfers were effected and the lecithin was spread over 100 tubes, which is near the capacity of the machine.

Gas-liquid chromatography. The technique used was as described by Collins (1963), and quantitative results with the National Heart Institute (Bethesda, Md., U.S.A.).

Fatty Acid Standards A, B, C and D agreed with the stated composition data with a relative error less than 5% for major components (more than 10% of total mixture) and less than 10% for minor components (less than 10% of total mixture). The procedure described by Collins (1963) was used for the preparation of the methyl esters of the fatty acids.

Determination of fatty acids at the 2 position of lecithin. The contents of the tube to be analysed, after removal of the solvent, were dissolved in diethyl ether so that the concentration was 2.0 mg./ml., the amount of lecithin being interpolated from neighbouring tubes. The solution of black-snake venom was added in the proportion of 0.10 ml. for each 3 ml. of diethyl ether. The mixture was allowed to react for 1 hr. at 25° and the solvent and water were removed by distillation *in vacuo* at the end of this time. A solution of diazomethane (100–200 μ moles) in dry diethyl ether was added and the solution left for 30 min. The excess of reagent and the solvent were finally removed *in*

vacuo, and the methyl esters distilled on to a 'cold finger' in the same way as in the normal preparation of fatty acid methyl esters for gas-liquid chromatography. Neither free fatty acids nor methyl esters of the fatty acids could be detected in the residues.

Determination of fatty acids at the 3 position of lecithin. An attempt was made to determine the fatty acids at the 3 position by treating the residue, after removal of the fatty acids released by the phospholipase A, by using the procedure described by Hanahan, Brockerhoff & Barron (1960) in which the lecithins and lysolecithins were dissolved in absolute ethanol at –25° and the lysolecithins were precipitated by the addition of diethyl ether at this temperature.

RESULTS

Lecithin-sphingomyelin fraction. Two rats weighing 310 g. and 312 g. yielded 24 g. of liver containing 2.08 g. of lipids, from which 310 mg. of lecithins was isolated. Thin-layer chromatography showed sphingomyelins and traces of lysolecithins in this fraction of lecithins. The fatty acid composition of this fraction is shown in Table 1; fatty acids with molecular weights greater than arachidonic acid were present to less than 1%.

The countercurrent distribution with 400 transfers involved discarding fractions free of lecithin. This was accomplished partly by calculation from previous distributions and by testing borderline fractions by the use of thin-layer chromatography. Fractions 250–270 still contained sphingomyelins but in amounts less than 10% of the lecithins and in any case these tubes were not included in the analysis.

Analysis of lecithins. The contents of every fifth tube was analysed for phosphorus and radioactivity, the contents of the adjacent tubes were analysed for total fatty acids and a third series of tubes were

Table 1. *Fatty acid composition of the total lecithins*

A sample of the total lecithins was subjected to methanolysis and the methyl esters of the fatty acids were analysed by gas-liquid chromatography. $\Delta^{6,9}$ -Octadecadienoic acid and $\Delta^{5,8,11}$ -eicosatrienoic acid were identified from their relative retention times.

Fatty acid	Amount (moles/ 100 moles)
Palmitic acid*	21.9
Palmitoleic acid*	9.4
Stearic acid*	17.1
Oleic acid*	30.6
Linoleic acid*	1.8
$\Delta^{6,9}$ -Octadecadienoic acid	1.3
$\Delta^{5,8,11}$ -Eicosatrienoic acid	16.1
Arachidonic acid*	1.8
	100.0

* Identified by comparison with authentic samples.

Table 2. *Fatty acid composition of lecithins distributed in carbon tetrachloride-methanol-water*

The methyl esters of the fatty acids were prepared from the total lipids in the tubes shown and analysed by gas-liquid chromatography. Experimental details are given in the text. —, Fatty acids not detected.

		Amount (moles/100 moles)														
Tube no....	...	174	179	184	189	194	199	204	209	214	219	224	229	234	239	244
Myristic acid*		—	—	—	—	—	—	—	—	—	0.2	0.5	0.8	1.1	1.6	2.0
Palmitic acid*		8.8	4.2	2.5	2.8	3.7	8.2	14.7	21.4	27.2	32.4	37.0	38.2	38.9	36.4	36.1
Palmitoleic acid*		1.7	1.1	0.5	0.4	—	0.5	2.3	4.2	7.5	12.5	19.0	24.0	28.5	34.5	36.7
Stearic acid*		49.1	47.5	46.5	42.7	36.0	29.8	22.2	17.0	11.8	7.8	5.0	4.4	4.4	4.1	5.6
Oleic acid*		12.4	15.6	18.8	21.9	27.5	31.8	35.1	37.2	37.2	34.2	29.5	25.0	20.6	15.2	13.2
Linoleic acid*		2.5	—	1.5	1.1	—	1.7	2.1	2.3	2.0	1.3	1.9	2.5	2.3	1.9	1.2
$\Delta^{6,9}$ -Octadeca- dienoic acid		2.6	—	—	0.6	—	—	1.4	1.2	1.7	2.0	2.2	2.6	2.4	2.1	1.2
$\Delta^{5,8,11}$ -Eicosatri- enoic acid		22.7	29.0	29.0	28.5	29.4	25.7	19.2	15.1	11.3	8.4	5.0	2.5	1.9	4.2	4.0
Arachidonic acid*		—	2.8	1.2	2.0	3.4	2.3	3.0	1.6	1.3	1.3	0.6	—	—	—	—

* Identified by comparison with authentic sample.

Table 3. *Fatty acid composition at the 2 position of lecithins distributed in carbon tetrachloride-methanol-water*

Fatty acids at the 2 position of lecithins were hydrolysed with phospholipase A and the liberated fatty acids converted into methyl esters, separated from the unhydrolysed material and analysed by gas-liquid chromatography. Experimental details are given in the text. —, Fatty acids not detected.

			Amount (moles/100 moles)													
Tube no....	...	...	176	183	188	193	198	203	208	213	218	223	228	233	238	243
Palmitic acid*			11.2	4.4	1.7	1.7	1.7	2.0	3.7	3.8	4.3	5.8	6.4	8.0	9.5	10.1
Palmitoleic acid*			—	—	—	0.8	1.1	2.5	4.5	10.3	14.6	22.0	31.1	39.1	51.0	64.6
Stearic acid*			9.5	2.8	1.5	2.4	1.7	1.5	1.2	1.1	0.9	0.8	0.7	1.0	1.0	1.8
Oleic acid*			25.8	21.5	27.7	30.0	34.0	39.0	44.0	47.6	49.5	48.0	42.0	36.8	23.1	16.7
Linoleic acid*			—	—	—	0.2	3.2	4.1	4.2	4.5	4.9	4.8	4.4	4.8	2.1	2.2
$\Delta^{6,9}$ -Octadecadienoic acid			—	—	—	0.1	1.7	2.8	2.8	3.0	4.0	5.0	4.5	5.5	3.3	3.3
$\Delta^{5,8,11}$ -Eicosatrienoic acid			53.4	65.6	63.1	56.4	51.8	42.0	35.0	25.1	19.0	11.0	6.6	4.3	3.2	—
Arachidonic acid*			—	5.7	6.0	7.3	4.8	4.1	3.6	3.3	2.8	2.0	0.9	1.4	—	—

* Identified by comparison with authentic sample.

used for the determination of the fatty acids situated at the 2 position of the lecithin. The results of these analyses are shown in Fig. 1 and Tables 2 and 3.

It had been hoped that the residue obtained after removing the fatty acids liberated by phospholipase A could be used to obtain the fatty acids present in the 3 position. The separation described in the Experimental section was carried out and the precipitated lysolecithins were examined for fatty acids. Comparison of the results obtained by the analysis of the lysolecithins with the figures obtained by difference (see below) showed that the recoveries of palmitic acid and stearic acid were quantitative, average recovery 99% (tubes 198–243). However, the recovery of the palmitoleic acid and oleic acid was low and averaged 56% (tubes 203–243) for palmitoleic acid and 45% (tubes 186–223) for oleic acid. Outside the range of tubes shown, the quantities were small and the errors consequently are larger.

The amounts of the fatty acids in the 3 position were calculated by difference as follows. The percentage of each fatty acid in the total mixture in each tube was plotted against tube number and a curve drawn through the points; this curve was used to interpolate the percentage in the tubes on which the determination of the 2 fatty acids had been made. The remainder of the calculation refers to this series of tubes and the percentage of a fatty acid in position 3 was obtained as the difference between twice the percentage of this fatty acid in the total mixture and the percentage of that at position 2. From these figures the total amount of each fatty acid in the 2 or 3 position could be calculated from the amount of phosphorus as

Fig. 1. Countercurrent distribution of lecithins from the livers of two rats deficient in essential fatty acids. The solvent system was carbon tetrachloride-methanol-water and 400 transfers were effected. The radioactive phosphorus was administered 1 hr. before death. —, Distribution of phosphorus; ----, counts/min.

interpolated from Fig. 1 and the results are shown in Figs. 2 and 3.

These results are similar to those obtained by Collins (1963) in that the distribution of lecithins containing palmitic acid differs from those of lecithins with stearic acid as shown in Table 4. The ratio of the partition coefficients of palmitic acid to that of stearic acid is the same in both experiments and the absolute change is presumably due to small differences in the solvent mixture and temperature. The distribution of radioactivity follows that of palmitic acid very closely and it can be calculated by the method of least squares that the average specific radioactivity of the lecithins

Fig. 2. Countercurrent distribution of lecithins showing the amounts and nature of the fatty acids esterified at the 3 position. The distribution is the same as shown in Fig. 1 and the fatty acids were determined after hydrolysis of the lecithin with phospholipase A. —, Stearic acid; ----, palmitic acid; ·—·—, palmitoleic acid; ·····, oleic acid.

Fig. 3. Countercurrent distribution of lecithins showing the amounts and nature of the fatty acid esterified at the 2 position. The distribution is the same as shown in Fig. 2 and the fatty acids were determined after hydrolysis of the lecithin with phospholipase A. —, Stearic acid; ----, palmitic acid; ·—·—, palmitoleic acid; ·····, oleic acid; — · — · —, linoleic acid; — · — · — · —, $\Delta^{6,9}$ -octadecadienoic acid; — · — · — · — · —, $\Delta^{5,8,11}$ -eicosatrienoic acid; — · — · — · — · — · —, arachidonic acid.

Table 4. Comparison of the partition coefficients of palmitic acid and stearic acid and of radioactivity

The partition coefficient was calculated from the number (n) of the tube with the maximum content of a component by the use of the formula $n(400-n)$.

	Collins (1963)	This paper	Ratio
Palmitic acid	1.37	1.18	1.16
Stearic acid	1.07	0.96	1.11
Radioactivity	1.25	1.16	1.08
Ratio of partition coefficient of palmitoyl to that of stearoyl lecithins	1.28	1.23	

not containing palmitic acid is 356 ± 100 counts/min./ $\mu\text{g. atom}$ of phosphorus and that of the palmitoyl lecithins is 5950 ± 72 counts/min./ $\mu\text{g. atom}$ of phosphorus. A comparison of Figs. 2 and 3 suggests that most of the $\Delta^{5,8,11}$ -eicosatrienoic acid and arachidonic acids occur as the stearoyl lecithin or oleoyl lecithin. A striking difference from the results obtained by Collins (1963) for the normal rat is that a quarter of the fatty acids in the 3 position are unsaturated.

DISCUSSION

As in the previous investigation (Collins, 1963), the nature of the diet minimized the number of fatty acids present and this facilitated the study. Further, the method for separating the lecithins gave a quantitative yield, thus ensuring that none of the lecithins was lost because of differences in solubility or unequal adsorption.

In the determination of the fatty acids at the 3 and 2 positions it was important that the results were quantitative and specific. In all the fractions analysed thin-layer chromatography had shown that lecithins were the only lipid materials present and hence the determination of total fatty acids presented no difficulty. In the procedure described in this paper for the determination of the fatty acids at the 2 position by the use of phospholipase A the extent of hydrolysis was greater than 90%. The separation of the liberated fatty acids (as their methyl esters) was quantitative as judged by using thin-layer chromatography for testing the residue after removal of the methyl esters by sublimation.

The method of determining the fatty acid at the 3 position by difference is justified by the fact that lecithins only were present and by the absence of plasmalogens. Thus the main source of error is in the gas-liquid chromatography. A recent discussion of this (Horning *et al.* 1964) emphasizes

Table 5. *Comparison of the distribution of saturated and unsaturated fatty acids between the 3 and 2 positions*

These figures were obtained by the use of phospholipase A and the analysis of the liberated fatty acids and lysolecithins.

Sample	Authors	Percentages			
		3 position		2 position	
		Satd.	Unsatd.	Satd.	Unsatd.
Rat liver lecithins	Collins (1963)	99	< 1	< 1	99
Rat liver lecithins	This paper	73.8	26.2	5.4	94.6
Egg lecithins	Menzel & Olcott (1964)	86	14	6	94
Rat liver lecithins	Menzel & Olcott (1964)	82	18	3	97
Ox liver lecithins	Menzel & Olcott (1964)	66	34	1	99
Egg lecithins	Kai, Joshita & Saga (1963)	96.2	3.8	9.5	90.5
		95.2	4.8	5.1	94.9
Ox liver lecithins	Kai <i>et al.</i> (1963)	77.6	22.4	20.3	79.6
		77.4	22.6	30.3	69.7
Pig serum lecithins	Leat (1964)	74.6	25.4	3.8	96.2

Table 6. *Comparison of the distribution of saturated and unsaturated fatty acids between different types of lecithin*

These figures were obtained either by the procedure used in this paper, or by the oxidation of the total mixture and separation into groups (Privett & Black, 1961), or by measuring differential hydrolysis rates with phospholipase A (Moore & Williams, 1964).

Sample	Authors	Percentages			
		Di-satd.	3 unsatd. 2 satd.	3 satd. 2 satd.	Di-unsatd.
Egg lecithins	Privett & Black (1961)	4.5	4.9	87.6	3.0
Egg lecithins	Moore & Williams (1964)	0.5	3.8	93.9	1.8
Rat liver lecithins	Collins (1963)	~1	~1	99	~1
Rat liver lecithins	This paper	5.0	0.5	74.68	21.26

that the errors are larger than often realized and can vary widely between different Laboratories. For these reasons the Fatty Acid Standard Mixtures issued by the National Heart Institute, Bethesda, Md., U.S.A., have been used to check the instrument used in this work. Because of the larger errors in determining some of the minor components the analyses were at least duplicated.

Recent work has shown that the findings of Tattre (1959) and of Hanahan *et al.* (1960) that the 2 position of lecithins was occupied exclusively by unsaturated fatty acids and the 3 position only by saturated fatty acids is not in fact entirely true. Results by Menzel & Olcott (1964) show that in rat liver lecithins there were 18% unsaturated fatty acids in the 3 position. Tables 5 and 6 give a comparison of recent findings which show that the 2 position has at least 70% unsaturated fatty acids but the 3 position can have as much as 34%. One can thus conclude that the present results are consistent with the findings in other Laboratories and that the result described by Collins (1963) appears

Table 7. *Specific radioactivities of the liver lecithins*

A comparison with the results of Collins (1963), in which the specific radioactivity of the inorganic phosphate in the liver is put equal to 100. The $H_3^{32}PO_4$ was administered 1 hr. before death.

	Normal rats	Deficient rats
Lecithins not containing palmitic acid	0.75	0.91
Palmitic acid (%)	21.3	21.9
Oleic acid (%), 3 position	—	21.4
Oleic acid (%), 2 position	16.8	39.8
Partition coefficient of oleic acid, 3 position	1.11	1.13
Partition coefficient of palmitic acid	1.40	1.47
Partition coefficient of the radio-activity	1.29	1.20

to be an extreme case where, because of perhaps a dietary accident, the distribution of fatty acids between the 3 and 2 position is close to that first

postulated. The finding that the eicosatrienoic acid occurs predominantly in the 2 position agrees with Leat (1964), who examined pig serum lecithins in animals lacking linoleic acid in their diet.

As in the normal rats (Collins, 1963), the palmitoyl lecithins from deficient animals contain more labelled phosphorus than those not containing palmitic acid. Table 7 shows that the specific radioactivity, relative to that of the inorganic phosphate, of the lecithins containing palmitic acid and derived from deficient animals is double that found in normal animals. The proportion of palmitic acid is the same and thus this increase of specific radioactivity can be due either to an increase in the turnover rate of one or more lecithins or to an increase in the amount of one or more lecithins but with no increase in the rate of turnover. This cannot be decided but the large increase in the percentage of oleic acid in the 2 position in the lecithins from deficient animals and the absence of an increase of any other major fatty acid with a partition coefficient near to 1.20 suggests that palmitoyl oleoyl lecithin may be the lecithin with the most label.

Collins (1962) suggested that the increase in the turnover of liver lecithins in rats deficient in essential fatty acids as compared with normal animals was due to the lowered amount of arachidonic acid. While this may be the underlying reason the direct cause of the increased specific radioactivity of the lecithins seems to be an increase

in a palmitoyl lecithin, possibly 3-palmitoyl 2-oleoyl lecithin.

Thanks are due to Mrs E. Rotenberg for her technical assistance and to the National Health and Medical Research Council, Commonwealth of Australia, for financial support.

REFERENCES

- Collins, F. D. (1959). *Biochem. J.* **72**, 532.
Collins, F. D. (1962). *Biochem. biophys. Res. Commun.* **9**, 289.
Collins, F. D. (1963). *Biochem. J.* **88**, 319.
Collins, F. D. & Wheeldon, L. W. (1957). *Biochem. J.* **66**, 441.
Doery, H. & Pearson, J. (1961). *Biochem. J.* **78**, 820.
Galanos, D. S. & Kapoulas, V. M. (1962). *J. Lipid Res.* **3**, 134.
Hanahan, D. J., Brockerhoff, H. & Barron, E. J. (1960). *J. biol. Chem.* **235**, 1917.
Horning, E. C., Ahrens, E. H., Lipsky, S. R., Mattson, F. H., Mead, J. F., Turner, D. A. & Goldwater, W. H. (1964). *J. Lipid Res.* **5**, 20.
Kai, M., Joshita, T. & Saga, M. (1963). *J. Biochem., Tokyo*, **54**, 403.
Leat, W. M. F. (1964). *Biochem. J.* **91**, 444.
Menzel, D. B. & Olcott, H. S. (1964). *Biochim. biophys. Acta*, **84**, 133.
Moore, J. H. & Williams, D. L. (1964). *Biochim. biophys. Acta*, **84**, 41.
Privett, O. S. & Black, M. L. (1961). *J. Lipid Res.* **2**, 37.
Tattre, N. H. (1959). *J. Lipid Res.* **1**, 60.
Wheeldon, L. W. & Collins, F. D. (1957). *Biochem. J.* **66**, 435.