Precision of Extraction, Fractionation, and Fatty Acid Analysis of Rat Liver Lipids and Stability of Fatty Acids During Storage

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Lipids were extracted from freeze-dried liver with a coefficient of variation of 1%. Separation of lipids on acid-washed Florisil yielded a neutral lipid and two phospholipid fractions with relative errors of 2% for each fraction, but fractionation of neutral lipids on unwashed Florisil was less precise. Samples of freeze-dried liver or of lipid extracted from liver tissue were stored at −30 °C. After 2 weeks, the fatty acid compositions of neutral and lecithin lipids were essentially unchanged but that of cephalin lipids had changed significantly. Changes in lipid composition continued rather slowly through 8 weeks of storage but rapidly between 8 and 12 weeks. Changes in fatty acid composition during storage were less apparent when calculated as percent of total fatty acids rather than in absolute quantity, and were less evident when total lipids rather than lipid fractions were analyzed.

Detailed analysis of lipids from biological sources involves extraction, fractionation into several classes, and quantitation of the fractions and of their fatty acid components. Such an analysis requires a considerable length of time so that if a significant number of samples are to be analyzed, some must be stored for a while before the work is completed. Many authors indicate this by reporting the temperature at which samples are held until analysis and sometimes even state the maximum duration of storage. However, the only reports that we found in the literature concerning alterations in lipids during storage were related to organoleptic quality of food products. Another problem encountered in lipid analysis of biological materials, such as liver, is that the lipid is not evenly distributed throughout the tissue. If the whole organ cannot be used for the assay, then some method must be adopted for obtaining a representative sample of the tissue.

Some of our studies were concerned with effects of dietary treatments on lipid composition of animal tissues (Miller, 1974; Landes and Miller, 1974). Since several diets were fed concomitantly to groups of 10–15 rats, it was necessary either to obtain tissues from all of the animals within a short time period and hold them before analysis or to analyze tissues from a few animals at the time while the remaining rats were maintained for prolonged intervals on the diets. It seemed that the objectives of our studies would be better served by feeding all of the animals for the same time period and storing samples as necessary before analysis.

Quick freezing of the organs after autopsy followed by lyophilization appeared to be the most practical course to minimize post-mortem changes in lipid composition and to provide a fairly homogeneous sample from which representative aliquots could be taken. Having accepted the compromise of lyophilized tissue as our starting material, we wished to evaluate the precision with which several steps in the lipid analysis scheme could be accomplished. A second objective was to determine how long the material could be stored before lipid composition changed enough to interfere with interpretation of data on effects of dietary treatments on tissue lipids.

Bligh and Dyer (1959) indicated that lipid could be extracted from fresh cod muscle by their procedure with a high degree of reproducibility. Carroll (1961, 1963) reported quantitative recovery of standard lipid materials from Florisil columns, but did not report on the precision of separation of natural lipids. In a recent review of lipid methodology, Privett et al. (1971) indicated a preference for acid-washed Florisil for fractionation of lipid extracts and reported essentially complete recovery of neutral and phospholipids from the original sample. Reproducibility of the procedure for transesterification and gas–liquid chromatography of fatty acids was reported by Worthington et al. (1972b). The reproducibility of the extraction procedure and of separations of lipid classes on Florisil was reassessed in our laboratory. In addition, the total variability to be expected from the summation of the procedures for determining fatty acid composition of various lipid fractions can be assessed from the data presented.

METHODS

Liver Samples. Rats which had been fed laboratory chow and deionized water ad libitum were anesthetized with diethyl ether and exsanguinated by heart puncture. The livers were removed, washed in deionized water, blotted dry, and frozen overnight. They were freeze-dried, ground through a 20 mesh screen, and thoroughly mixed. Sufficient 1-g samples for the entire storage study were weighed, placed in individual vials, and held at −30 °C until the predetermined sampling date. Lipid was extracted from another portion of the dried liver sample and made into one composite from which a number of aliquots were taken. Solvent was evaporated from these samples under nitrogen and they also were stored at −30 °C until needed. The samples used to assess the variability of the extraction procedure and of chromatography on unwashed Florisil were similarly prepared from two different groups of rats. All solvents used in this study were redistilled from glass apparatus.

Lipid Extraction. Lipid was extracted from the samples by a procedure similar to that described by Bligh and Dyer (1959). Each 1-g sample was extracted initially in a mixture of 20 ml of methanol, 10 ml of chloroform, and 8 ml of water in a semi-micro stainless steel blender jar. Subsequently 10 ml of chloroform and then 10 ml of water were added. The samples were centrifuged at 4 °C and the chloroform layer removed with a pipet. Another 20 ml of chloroform was added to the sample and after shaking and centrifugation, this chloroform was also removed by pipet. The chloroform extracts were filtered through anhydrous sodium sulfate which had been previously wet with chloroform, and were washed through with a final portion of the same solvent. The solvent was removed on a rotary vacuum evaporator with the water bath held at about 60 °C and the sample was taken up in hexane. Samples to be chromatographed in Florisil columns were transferred to small vials and the hexane evaporated under nitrogen. The samples on which fatty acid content of the total lipid was to be determined were

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made to 10-ml volume and a 2-ml aliquot was taken for methylation. Larger samples were extracted with proportionate increases in volumes of solvents used.

Lipid Fractionation. Florisil, screened to 60–80 mesh size, was washed with hydrochloric acid as described by Carroll (1963). Columns 1 cm in diameter were packed to a height of 20 cm with the washed Florisil. The columns were filled with hexane and the Florisil was run in slowly from a separatory funnel while the column was tapped gently. The hexane was allowed to run down to the top of the Florisil and the sample (100–200 mg of lipid), in a small quantity of hexane, was put on the column and washed in with 15 ml of hexane. Neutral lipids, containing glycerides, free fatty acids, cholesterol, and its esters, were eluted with 150 ml of 25% diethyl ether in hexane; one phospholipid fraction, containing primarily phosphatidylethanolamines (cephalin), was eluted with 150 ml of 18% methanol in chloroform; and the second phospholipid fraction, consisting mostly of phosphatidylcholines (lecithin), was eluted with 100 ml of methanol. Solvent was evaporated from these fractions as described above. For gravimetric analyses the samples were collected in tared vials and held under vacuum for about 2 h before weighing.

Neutral lipids were separated into four fractions on unwashed Florisil screened to 80–100 mesh. Columns were packed as described above and the sample (100–200 mg of lipid) was washed on with 20 ml of hexane. Cholesterol esters were eluted with 35 ml of 5% diethyl ether in hexane; triglycerides were eluted with 50 ml of 15% diethyl ether in hexane; cholesterol (and any di- or monoglycerides present) was eluted with 50 ml of 2% methanol in ether and, finally, free fatty acids were eluted with 4% acetic acid in ether. The samples were prepared for gravimetric analysis as described above. Separations on Florisil were monitored by thin-layer chromatography using plates coated with silica gel G. Hexane–diethyl ether–acetic acid (90:10:1) was used to separate neutral lipids and chloroform–methanol–water (65:25:3) was used to separate phospholipids.

Gas–Liquid Chromatography. The samples were transesterified as described by Worthington et al. (1972a), and henicosanoic acid was added as an internal standard. Gas chromatographic determination of the methyl esters was accomplished using a Micro-Tek 220 unit equipped with a flame ionization detector and Infotronics CRS-208 integrator. The injection port was maintained at 270 °C and the detector at 275 °C and the helium flow rate was 100 ml/min. Glass columns 6 ft × 0.25 in. o.d. were packed with 80–100 mesh Chromasorb W (AW, DMCS) coated with 20% EGS stabilized with 3% phosphoric acid. The column was operated at 190 °C. Peak identification was made by comparison of retention times with authentic standards run under identical conditions or with published values. Data were calculated both as percent distribution of the fatty acids by comparison of the area under each peak with the total area under all peaks and as absolute quantity of the acids by comparison of peak areas with that of the internal standard.

Treatment of Data. All data were subjected to analysis of variance and significant differences among the means in the storage study were determined using Duncan's multiple range test (Duncan, 1955). Fatty acid data are average values for three samples each of lipid extract and freeze-dried tissue. The standard deviation calculated from the pooled error variance was used to estimate the coefficient of variation.

RESULTS AND DISCUSSION

Lipid Extraction. Lipid was extracted from 10 1-g samples as described above and measured gravimetrically. A mean of 129 mg of lipid per sample with a coefficient of variation of 1.01% was obtained. This is in good agreement with the work of Bligh and Dyer (1959) who obtained a relative standard error of 2% for extraction of lipid from fresh cod muscle.

Table I. Reproducibility of Separations on Florisil Columns

	mg/g of dried liver		Coeff of variation, %
	Mean	Std dev	
Unwashed Florisil, 9 Samples			
Cholesterol esters	24.5	2.59	10.55
Triglycerides	68.0	4.03	5.95
Cholesterol	15.3	0.89	5.85
Free fatty acids	8.97	0.87	9.71
Acid-Washed Florisil, 10 Samples			
Neutral lipids	65.2	1.18	1.82
Cephalin	33.0	0.59	1.80
Lecithin	61.8	1.35	2.18

Lipid Fractionation. Separation of neutral lipids on unwashed Florisil into the classes listed in Table I was clear-cut, with no overlapping of the classes that could be detected by thin-layer chromatography. Quantification of the triglyceride and cholesterol fractions was fairly good with coefficients of variation of about 6%, but the gravimetric measurement of the cholesterol ester and free fatty acid fractions was rather imprecise with about 10% variability. For these separations the Florisil was used as received from the supplier except that the original 60–100 mesh product was resieved and the fraction measuring 80–100 mesh was used. Though the samples were put on the columns with 20 ml of hexane which was discarded, the cholesterol ester fraction was the first collected and may have contained column impurities. Failure to remove all of the solvent from the samples may have contributed to the variation also, especially the acetic acid used in elution of the free fatty acids from the columns. Holding the sample under vacuum for 2 h improves the precision over that obtained just by evaporating the solvents with a stream of nitrogen.

The separations into one neutral lipid and two phospholipid fractions achieved with acid-washed Florisil were highly reproducible as indicated by the very low coefficients of variation obtained (Table I). Thin-layer chromatography indicated that neutral lipids were completely separated from phospholipids and that phosphatidylethanolamine and phosphatidylcholine were separated from each other.

Precision of Fatty Acid Analysis. The precision with which fatty acid composition of rat liver samples can be measured by the system employed in this study is indicated by the coefficients of variation presented in Tables II, III, and IV. When composition is expressed as distribution of each fatty acid within the total quantity of fatty acids present in the sample, those acids which contribute as much as 1% of the total acids of the unfractionated lipid samples were measured with a variation of less than 5%. This same degree of precision was obtained in measurement of fatty acids in the samples separated on acid-washed Florisil except for quantitation of palmitoleic acid in the two phospholipid fractions (Tables III and IV) and one of the docosapentaenoic acids in the cephalin fraction (Table IV).

When the data were calculated as absolute quantity of each fatty acid in the sample by comparison with an internal standard, those fatty acids which contributed as much as 0.5 mg to the sample were measured with a variation of less than 5% of the mean with a few excep-

Table II. Fatty Acid Composition of Total Lipid of Liver Samples Stored at −30 °C[a]

Weeks stored	Fatty acid, mg/g of dried liver									
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:6	Total
					Lipid Extract					
0	0.71	23.5^{b}	4.4^{b}	12.6	27.4	15.7^{ab}	0.94^{a}	16.1	5.5	112^{b}
1	0.71	26.0^{ab}	4.6^{ab}	13.1	27.6	16.0^{a}	0.98^{a}	16.3	5.5	116^{ab}
2	0.72	28.1^{a}	4.7^{a}	13.3	28.0	16.1^{a}	0.95^{a}	16.2	6.0	119^{a}
4	0.72	28.0^{a}	4.7^{a}	13.0	27.3	15.6^{ab}	0.84^{b}	16.1	5.4	116^{ab}
8	0.72	27.2^{a}	4.8^{a}	12.8	26.8	15.3^{b}	0.84^{b}	15.7	5.3	113^{b}
12	(0.70)	(27.5)	(5.2)	(13.1)	(27.9)	(16.1)	(0.79)	(16.6)	(6.8)	(121)
Mean	0.72	26.6*	4.7**	12.9	27.4	15.7*	0.91**	16.1	5.5	115*
CV, %	3.32	6.1	1.9	3.3	2.1	1.8	4.17	1.9	4.7	2.0
					Freeze-Dried Tissue					
0	0.72	26.0	4.6	12.6	26.9	15.6	0.97^{a}	16.2	5.8	114
1	0.66	26.3	4.5	12.7	27.2	15.7	0.92^{a}	16.0	5.4	114
2	0.71	27.6	4.8	12.6	27.5	15.7	0.88^{ab}	15.8	5.3	115
4	0.70	27.0	4.7	12.5	26.9	15.5	0.85^{ab}	15.4	5.3	112
8	0.67	26.7	4.6	12.5	26.1	14.9	0.78^{b}	15.3	5.3	109
12	(0.66)	(25.8)	(5.0)	(12.1)	(26.3)	(15.1)	(1.03)	(15.5)	(6.1)	(115)
Mean	0.69	26.7	4.6	12.6	26.9	15.5	0.88**	15.8	5.4	113
CV, %	4.11	4.1	3.4	2.8	2.6	2.8	5.35	2.6	5.6	2.0
					% of total fatty acids					
					Lipid Extract					
0	0.64	21.0^{b}	4.0^{b}	11.3	24.4	14.1^{a}	0.85^{a}	14.4^{a}	4.9	
1	0.62	22.4^{ab}	4.0^{b}	11.2	23.8	13.8^{ab}	0.85^{a}	14.1^{ab}	4.8	
2	0.60	23.9^{a}	3.9^{b}	11.2	23.5	13.5^{b}	0.80^{ab}	13.7^{b}	5.0	
4	0.62	24.1^{a}	4.0^{b}	11.2	23.6	13.4^{b}	0.72^{b}	13.8^{b}	4.6	
8	0.64	24.2^{a}	4.3^{a}	11.4	23.5	13.6^{ab}	0.75^{b}	14.0^{ab}	4.7	
12	(0.59)	(23.3)	(4.4)	(11.1)	(23.6)	(13.6)	(0.67)	(14.0)	(5.8)	
Mean	0.62	23.2*	4.0**	11.2	23.8	13.7**	0.79**	14.0*	4.8	
CV, %	3.72	5.0	0.5	2.1	1.6	1.4	4.46	1.9	3.8	
					Freeze-Dried Tissue					
0	0.63^{a}	22.7	4.0^{c}	11.0^{b}	23.8	13.6	0.84^{a}	14.2^{a}	5.0	
1	0.59^{b}	23.0	3.9^{c}	11.1^{ab}	23.8	13.8	0.81^{ab}	14.0^{ab}	4.7	
2	0.62^{a}	24.0	4.2^{b}	11.0^{b}	23.9	13.6	0.76^{ab}	13.5^{c}	4.6	
4	0.63^{a}	23.9	4.2^{b}	11.1^{ab}	23.9	13.7	0.78^{b}	13.7^{bc}	4.7	
8	0.63^{a}	23.8	4.3^{a}	11.4^{a}	23.7	13.7	0.72^{b}	14.0^{ab}	5.1	
12	(0.59)	(23.2)	(4.5)	(10.9)	(23.6)	(13.6)	(0.89)	(13.9)	(5.4)	
Mean	0.62*	23.5	4.1**	11.1*	23.8	13.7	0.78**	13.9*	4.8	
CV, %	2.46	3.1	1.4	1.6	1.5	1.5	4.37	1.7	4.7	

[a] Means within a column for each lipid preparation having no common superscript are significantly different at $P < 0.05\%$ (*) or $P < 0.01\%$ (**). Data from samples stored for 12 weeks were not included in the statistical analysis.

tions. Notable among these exceptions is imprecision of palmitic acid in the total lipid samples stored as a lipid extract and of docosahexaenoic acid in the freeze-dried composite sample (Table II). Generally speaking, the precision of measurement was poorer for fatty acids of the cephalin lipids than for those of the neutral lipids or lecithins.

Comparisons between the two methods of calculating the content of individual fatty acids in the samples indicate that in about 80% of the cases quantitation is more precise when composition is based on distribution of each fatty acid among the total content of fatty acids than when content is expressed as an absolute quantity of each acid in the sample relative to an internal standard.

The variation due to methodology should have been less in those samples obtained from a composite of lipid extracted from the freeze-dried liver than in those obtained from subsamples of the initial liver composite since one phase of the procedure, namely extraction of the lipid, was completed before subsampling occurred. The coefficients of variation for fatty acids of the total liver lipids listed in the first half of Table II indicate that this was the case except for palmitic and stearic acids. However, in data expressed as percent of total fatty acids there is less difference in variability between the two forms in which the samples were stored with some tendency for the samples stored as lipid extracts to be more variable. Since this difference is not likely to be attributable to sampling, it is probably an indication that the freeze-dried sample was more stable during storage than the lipid extracts.

Changes in Fatty Acids during Storage. Effects of storage on fatty acid composition of the samples are shown in Tables II, III, and IV. The gas–liquid chromatograms of samples stored for 12 weeks were clearly different from those of samples stored for shorter time periods. The most notable changes were the appearance of a broad peak in the area of the icosa- and docosapolyenoic acids, and a tendency for the baseline to be elevated in the region of the 18-carbon acids. Since these aberrations markedly increased the variability of this set of samples, they were omitted from the statistical calculations so that they would not mask more subtle changes in fatty acid composition that may have occurred prior to 12 weeks of storage. In samples stored for periods up to 8 weeks, only those changes which exhibit a trend with duration of storage are considered to be associated with storage per se. Other more random variations are assumed to be due either to heterogeneity in the original sample composite or to inability to reproduce technical procedures at the discrete time intervals involved in a storage study.

The most noticeable change in fatty acid composition, and one that would have been anticipated, is the reduction in linolenic acid. The quantity of this acid was significantly reduced in the total lipid fraction (Table II) after 4 weeks

Table III. Fatty Acid Composition of Neutral and Lecithin Fraction of Lipids of Freeze-Dried Liver Stored at −30 °C[a]

Weeks stored	Fatty acid, mg/g of dried tissue									
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:6	Total
					Neutral Lipid					
0	0.57^{a}	15.4^{a}	3.6^{a}	1.17	20.7^{a}	8.5^{a}	0.74^{a}	0.96		53^{a}
1	0.56^{ab}	14.7^{ab}	3.5^{a}	1.13	19.9^{a}	8.1^{ab}	0.74^{a}	0.97		52^{a}
2	0.55^{ab}	15.6^{a}	3.5^{a}	1.10	19.4^{a}	8.0^{b}	0.69^{ab}	0.96		52^{a}
4	0.53^{bc}	13.2^{b}	3.2^{b}	1.07	16.8^{b}	7.3^{c}	0.70^{a}	0.99		46^{b}
8	0.51^{c}	14.3^{ab}	3.2^{b}	1.14	17.9^{b}	7.3^{c}	0.62^{b}	0.95		47^{b}
12	(0.54)	(14.4)	(3.8)	(1.18)	(19.5)	(8.3)	(0.78)	(1.04)		(54)
Mean	0.54**	14.6**	3.4**	1.12	19.0**	7.8*	0.70**	0.97		50**
CV, %	2.90	4.0	2.6	4.11	2.6	2.3	3.95	3.87		2.4
					Lecithin Lipid					
0		7.9^{a}	0.54	7.4^{a}	3.8	4.0	0.10^{a}	10.0^{a}	2.5^{a}	38^{a}
1		7.5^{ab}	0.50	7.2^{a}	3.2	3.8	0.09^{ab}	9.6^{ab}	2.3^{bc}	37^{ab}
2		7.9^{a}	0.53	7.2^{a}	3.6	3.9	0.09^{ab}	9.7^{ab}	2.4^{ab}	37^{ab}
4		7.3^{b}	0.54	7.0^{ab}	3.4	3.7	0.08^{b}	9.2^{ab}	2.2^{c}	35^{ab}
8		7.2^{b}	0.58	6.8^{b}	3.4	3.7	0.08^{b}	9.0^{b}	2.3^{bc}	34^{b}
12		(7.7)	(0.75)	(7.4)	(3.8)	(4.0)	(0.11)	(10.3)	(2.6)	(39)
Mean		7.6*	0.54	7.1*	3.6	3.8	0.09*	9.5**	2.4*	37**
CV, %		3.8	7.42	2.7	3.7	3.3	7.94	3.2	3.1	30
	% of total fatty acids									
					Neutral Lipid					
0	1.08^{b}	29.0^{bc}	6.8	2.2^{bc}	38.9^{a}	16.1^{a}	1.39^{bc}	1.8^{b}		
1	1.08^{b}	28.3^{c}	6.7	2.2^{bc}	38.6^{a}	15.7^{abc}	1.44^{ab}	1.9^{b}		
2	1.06^{b}	30.1^{ab}	6.8	2.1^{c}	37.6^{b}	15.4^{c}	1.38^{bc}	1.9^{b}		
4	1.15^{a}	28.8^{bc}	7.0	2.3^{ab}	36.8^{b}	15.9^{ab}	1.53^{a}	2.2^{a}		
8	1.08^{b}	30.5^{a}	7.0	2.4^{a}	37.4^{b}	15.6^{bc}	1.31^{c}	2.0^{ab}		
12	(1.04)	(27.7)	(7.3)	(2.4)	(37.3)	(15.9)	(1.50)	(2.0)		
Mean	1.08**	29.4*	6.9	2.3*	38.0**	15.7*	1.40**	2.0		
CV, %	2.18	2.4	1.8	4.2	0.9	1.4	2.55	4.5		
					Lecithin Lipid					
0		20.6	1.4^{b}	19.3^{bc}	9.7	10.4	0.25	26.2	6.4^{b}	
1		20.4	1.4^{b}	19.6^{ab}	9.7	10.4	0.26	26.2	6.4^{b}	
2		21.0	1.4^{b}	19.2^{c}	9.6	10.3	0.24	25.8	6.4^{b}	
4		20.5	1.5^{ab}	19.8^{a}	9.7	10.4	0.24	26.1	6.4^{b}	
8		20.8	1.7^{a}	19.6^{ab}	9.9	10.6	0.23	26.0	6.7^{a}	
12		(20.0)	(1.9)	(19.1)	(9.9)	(10.5)	(0.28)	(26.7)	(6.6)	
Mean		20.7	1.5*	19.5*	9.7	10.4	0.24	26.1	6.5*	
CV, %		2.1	6.4	1.0	1.7	1.1	6.25	0.8	1.5	

[a] Means within a column for each lipid preparation having no common superscript are significantly different at $P < 0.05\%$ (*) or $P < 0.01\%$ (**). Data from samples stored for 12 weeks were not included in the statistical analysis.

Table IV. Fatty Acid Composition of Cephalin Fraction of Lipids of Freeze-Dried Liver Stored at −30 °C[a]

Weeks stored	Fatty acid, mg/g of dried tissue											
	16:0	16:1	18:0	18:1	18:2	20:4	22:0	22:4	22: 5n6	22: 5n3	22:6	Total
0	2.8^{b}	0.31^{c}	3.8	2.1^{a}	2.6^{c}	4.6	0.21	0.20	0.13	0.22	2.3	19.8^{c}
1	2.9^{b}	0.36^{bc}	3.8	2.5^{b}	2.8^{b}	4.5	0.22	0.18	0.13	0.20	2.2	20.3^{bc}
2	3.3^{a}	0.46^{a}	3.9	3.0^{a}	3.0^{a}	4.7	0.23	0.21	0.15	0.22	2.4	22.1^{a}
4	2.9^{b}	0.43^{ab}	3.6	2.7^{ab}	2.8^{b}	4.3	0.22	0.24	0.11	0.23	2.3	20.3^{bc}
8	3.2^{a}	0.48^{a}	3.9	3.0^{a}	2.9^{ab}	4.6	0.22	0.23	0.12	0.21	2.4	21.5^{ab}
12	(3.1)	(0.49)	(3.8)	(3.1)	(3.0)	(4.7)	(0.22)	(0.19)	(0.11)	(0.22)	(2.4)	(21.7)
Mean	3.0**	0.41**	3.8	2.7**	2.8**	4.5	0.22	0.21	0.13	0.22	2.3	20.8**
CV, %	2.0	6.97	4.3	3.8	2.0	4.0	5.01	10.2	11.6	10.2	3.7	2.3
	% of total fatty acids											
0	14.2^{b}	1.6^{b}	19.4^{a}	10.5^{c}	12.9^{b}	23.1^{a}	1.07	1.01^{ab}	0.66	1.1	11.7^{a}	
1	14.2^{b}	1.8^{ab}	18.8^{ab}	12.5^{b}	13.9^{a}	22.0^{ab}	1.07	0.87^{c}	0.62	1.0	10.6^{c}	
2	14.8^{a}	2.1^{a}	17.8^{b}	13.6^{ab}	13.8^{a}	21.3^{b}	1.03	0.93^{c}	0.68	1.0	10.7^{bc}	
4	14.2^{b}	2.1^{a}	17.8^{b}	13.6^{ab}	13.6^{a}	21.3^{b}	1.08	0.98^{bc}	0.63	1.2	11.3^{ab}	
8	14.8^{a}	2.2^{a}	18.2^{ab}	14.1^{a}	13.5^{a}	21.1^{a}	1.00	1.07^{a}	0.56	1.0	11.0^{b}	
12	(14.4)	(2.3)	(17.5)	(14.2)	(13.9)	(21.5)	(1.00)	(0.87)	(0.48)	(1.0)	(11.1)	
Mean	14.5*	2.0**	18.4**	12.9**	13.5**	21.8**	1.05	0.97**	0.63	1.0	11.1*	
CV, %	1.9	8.3	2.7	3.8	1.5	2.2	4.70	2.6	8.5	8.0	3.1	

[a] Means within a column not having a common superscript are significantly different at $P < 0.05\%$ (*) or $P < 0.01\%$ (**). Data from samples stored for 12 weeks were not included in the statistical analysis.

of storage as a lipid extract and after 8 weeks when stored as freeze-dried tissue. When the liver lipid was separated into several classes, changes in the fatty acid composition of neutral lipids (Table III) that were not apparent in the whole lipid sample became obvious. The quantities of the unsaturated acids, palmitoleic, oleic, and linoleic, were all

significantly reduced in the neutral lipids after 4 weeks of storage. The content of myristic acid in these lipids was also decreased during this time. These changes were reflected in a decrease in the total amount of fatty acids in the neutral lipids after 4 weeks' storage.

The total amount of fatty acids in the lecithin fraction obtained from the Florisil columns decreased during storage of the samples for 8 weeks. This loss was associated with reductions in the amount of palmitic, stearic, and arachidonic acids, which together comprise two-thirds of the total fatty acids in this lipid component.

The storage effects on the fatty acid composition of the cephalin fraction of the liver lipids are more ambiguous. There appears to be some increase in the total amount of fatty acids extracted in this fraction primarily as a result of increases in palmitic, oleic, and linoleic acids.

The loss of fatty acids from the neutral lipid fraction could be attributed primarily to oxidative degradation since most of the fatty acids lost had at least one double bond. The changes that occurred in the phospholipid fractions are not so easily explained. In viable liver tissue, fatty acids are readily interchanged among the various lipid classes, and phosphatidylethanolamines and phosphatidylcholines are interconverted by methylation and demethylation. However, very little enzymatic activity would be expected to occur in freeze-dried tissue held at −30 °C. Loss of saturated fatty acids from the lecithin fractions could have occurred by nonenzymatic hydrolysis of the acid attached to the α carbon of the glycerol moiety. Any free fatty acids thus formed should have appeared in the neutral lipids but the palmitic and stearic acid content of this lipid did not increase during storage. Separation of lipids into classes by column chromatography is an empirical procedure based primarily on the polarity of the lipids. The apparent increase in total fatty acids and several of the individual fatty acids in the cephalin fraction is probably due to changes in some lipids which altered their polarity and thus their pattern of elution from the Florisil column. This is further indicated by the fact that acids which appeared to increase in the cephalin fraction decreased in one or both of the other lipid classes.

When the data are calculated as percent distribution of each fatty acid within the total, the effects of storage tend to be obscured and many of the changes appear to be random variations in fatty acid composition. However, an exception to this is observed in the percentages of the monoenoic acids of the cephalin fraction during storage (Table IV) where marked increases occurred.

The data presented here indicate that rat liver samples can be stored at −30 °C for only short periods of time before significant changes occur in the fatty acid content of the lipids of the samples. Between 8 and 12 weeks of storage marked changes occurred which produced severe distortions in the data on fatty acid composition obtained by GLC analysis. These changes occurred in both freeze-dried liver samples and in extracts of the liver lipids and might possibly be attributed to depletion of some antioxidant present in the original sample. After 2 weeks of storage, fatty acid composition of the neutral and lecithin lipids was essentially unchanged from the original samples but some of the fatty acids of the cephalin lipid fraction had already changed significantly. The alterations that occurred in fatty acid composition of the lipids during storage might not be detected if the analyses are performed on total lipids of the samples rather than on the several fractions. The storage changes are also disguised in data calculated as distribution of fatty acids within the total fatty acid content of the samples rather than as the absolute quantity of each acid in the sample.

The precision with which the individual fatty acids were measured in the various lipid fractions was generally good despite the number of steps involved in preparation of the samples. Only in some of the docosapolyenoic acids of the cephalin fraction were the coefficients of variation greater than 10%. Separation of the extracted lipids into neutral and two phospholipid fractions on acid-washed Florisil was also highly reproducible.

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