would contain the same amount of "free" iron as the controls and that the remainder of the iron is in the gossypol-iron complex, then this yields a gossypol to iron ratio of 1.36 to 1. This approaches a 1:1 ratio which seems to be the most quoted value. If, on the other hand, it is assumed that all the gossypol in the blot is tied up in a 1:1 ratio complex with iron and that the remaining iron in the blot is "free," then statistical analysis shows the gossypol-treated sacs have a significantly lower "free" iron content ($P < 0.01$).

From the data presented herein, it is believed that gossypol reduced iron uptake of everted intestinal sacs by the formation of a gossypol-iron complex which is not readily absorbed.

Although gossypol forms a 1:1 molar complex with iron and this paper shows reduced tissue uptake of iron at a 1:1 molar ratio, feeding studies have shown that a 9.3:1 molar ratio of iron to gossypol is required to protect non-ruminants from gossypol toxicity (Smith and Clawson, 1970). It is felt that this is probably due to the heterogeneous nature of the chyme interfering with the intimate contact between iron and gossypol required for the formation of the complex.

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Effects of Several Vegetable Oils on Lipid Classes and Very Long Chain Polyenoic Fatty Acid Content of Rat Liver and Heart

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Weanling rats were fed diets containing 10% peanut, safflower, soybean, or linseed oil for 8 weeks to determine the effects of feeding natural fats varying widely in linoleic and linolenic acid content on the lipid classes and very long chain polyenoic fatty acid (VLCPFA) content of the liver and heart. Total liver lipid was reduced in the animals fed peanut oil and increased in the animals fed soybean oil compared to the other treatments, with these alterations being confined to the neutral lipids. The animals receiving the soybean and linseed oils accumulated more cephalin in relation to lecithin in the heart than the animals receiving the peanut and safflower oils. Fatty acid analyses indicated that the VLCPFA of the linolenic series increased in the phospholipids of the liver and heart with reductions in VLCPFA of the linoleic series as the dietary linolenic acid increased from trace amounts, and in the rats receiving linseed oil the VLCPFA of the linoleic series essentially disappeared with the exception of arachidonic acid which was greatly reduced.

Brain phospholipids of many species contain a relatively high concentration of docosahexaenoic acid (22:6, *n*-3), a metabolite of linolenic acid (Crawford, 1970). Fatty acids of the *n*-3 series have also been found in significant amounts in the lipids of the liver and heart (Egwim and Kummerow, 1972; Sinclair and Crawford, 1973). In studies conducted in our laboratory, it has been observed that when diets contained trace amounts of linolenic acid, docosahexaenoic acid was the only *n*-3 acid found in the heart and liver lipids with the remainder of the very long chain polyenoic fatty acids (VLCPFA), 20 or more carbons in the chain, being members of the linoleic acid (*n*-6) family. However, when oils containing more than trace amounts of linolenic acid were fed, docosapentaenoic (22:5, *n*-3) and eicosapentaenoic (20:5, *n*-3) acids were also present in these lipids.

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Table I. Percent Distribution of the Fatty Acids of the Dietary Oils

	16:0	16:1	18:0	18:1	18:2	18:3	Other[a]
Peanut	10.53	0.19	2.94	48.54	29.66	T[b]	8.14
Safflower	7.80	0.15	2.72	13.86	73.02	0.77	1.68
Soybean	11.14	0.16	4.00	23.75	51.10	7.55	2.30
Linseed	6.52	0.16	4.03	19.61	16.38	52.59	0.71

[a] Fatty acids included here were myristic acid and the 20-, 22-, and 24-carbon saturated and monounsaturated fatty acids along with traces of other fatty acids not identified. [b] Detected in amounts less than 0.1% using butane-1,4-diol succinate as liquid phase.

Holman and Mohrhauer (1963) have shown that there is nearly a tenfold preferential elongation and desaturation of linolenic compared to linoleic acid when near minimal concentrations of these purified acids were added to fat free diets. No information is available in the literature on the effects of natural oils varying widely in linoleic and linolenic acid content on the VLCPFA composition of the liver and heart. This study was undertaken to investigate the effects of several different vegetable oils containing different proportions of linoleic and linolenic acids fed at intermediate levels on VLCPFA composition of these tissues and on several other aspects of lipid composition.

MATERIALS AND METHODS

Weanling Sprague-Dawley male rats obtained from Charles River Breeding Laboratories were fed diets containing 10% oil from one of four different vegetable sources for 56 days. In addition to the oil each diet contained, in percent: casein 20.0; dextrin, 35.0; cellulose, 1.5; Vitamin Fortification Mixture (ICN Nutritional Biochemicals Corp.), 2.2; P-H Salt Mixture (ICN Nutritional Biochemicals Corp.), 4.0; zinc mixture (powdered sugar containing 1.35 mg of Zn as $ZnCO_3$ per g), 1.0; α-tocopherol, 0.0044; and sucrose to equal 100%. The oils used were peanut, safflower, soybean, and linseed oil. Each diet and water were fed ad libitum throughout the experimental period to seven animals.

Under ether anesthesia the animals were exsanguinated and serum was obtained from the clotted blood by refrigerated centrifugation. The livers, hearts, and epididymal fat pads were removed, cleaned of adhering tissue, washed, and weighed. The livers and hearts were frozen at −35° and lyophilized before analysis.

The lyophilized hearts from each treatment were pooled and composite samples of the lyophilized livers for each treatment were made. These samples were extracted by the method of Bligh and Dyer (1959) and the lipid was recovered. Solvent was evaporated from known portions of the extracts for determination of total lipid content of the tissues. Portions of these lipid extracts were applied to chromatography columns containing Florisil that had been acid washed as described by Carroll (1963) and the neutral lipid, containing glycerides, free fatty acids, and free and esterified cholesterol, was eluted with 25% diethyl ether in hexane. This was followed by elution of the cephalin fraction containing primarily phosphatidylethanolamine, with 18% methanol in chloroform, and finally elution of the lecithin fraction containing primarily phosphatidylcholine with 100% methanol. Portions of the liver extracts were applied to unwashed Florisil packed columns and the neutral lipid was separated in a manner similar to that described by Carroll (1961). The cholesterol esters were eluted with 5% diethyl ether in hexane, the glycerides and free cholesterol were eluted as one fraction with 2% methanol in diethyl ether, and the free fatty acids were eluted with 4% acetic acid in diethyl ether. All fractions were collected in tared vials for gravimetric measurements. The

Table II. Effects of Different Vegetable Oils on Food Intake, Body Weight, and Selected Tissue Components[a]

	Dietary oil			
	Peanut	Safflower	Soybean	Linseed
Food intake (g)	1140	1053	1135	1100
Body wt (g)	425	414	438	420
Epididymal fat pad wt (g)	4.80	3.66	4.48	4.04
Liver wt (g)	15.3	15.4	17.2	15.3
Moisture (%)	68.7	69.2	69.3	69.6
Lipid, total (%)	5.00	5.73	5.93	5.71
Neutral lipid (mg/100 g of tissue)	1781	2556	2806	2230
Cholesterol ester (mg/100 g of tissue)	149	288	499	278
Glycerides + cholesterol (mg/100 g of tissue)	1249	1832	1808	1596
Free fatty acids (mg/100 g of tissue)	384	436	499	360
Total cholesterol[b] (mg/100 g of tissue)	273	293	293	247
Cephalin (mg/100 g of tissue)	1180	1204	1179	1275
Lecithin (mg/100 g of tissue)	2034	1973	1942	2207
Heart wt (g)	1.12	1.14	1.12	1.15
Moisture (%)	78.2	77.7	77.8	78.1
Lipid, total (%)	3.26	3.25	3.25	3.16
Neutral lipid (mg/100 g of tissue)	449	457	464	420
Cephalin (mg/100 g of tissue)	1358	1390	1435	1428
Lecithin (mg/100 g of tissue)	1454	1406	1354	1317
Total cholesterol[b] (mg/100 g of tissue)	111	122	124	124
Serum cholesterol[b] (mg/100 ml)	165	161	158	143

[a] Values for lipid analyses are for pooled or composite sample, and values for other parameters are average values for seven animals. Analysis of variance was determined on the data where appropriate but differences due to dietary treatment were not significant at the 5% level of probability. [b] Total cholesterol was determined by chemical assay.

Table III. Concentration of Selected Fatty Acids in Liver Lipid Fractions of Rats Fed Different Vegetable Oils[a]

	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:4	20:5	22:5, *n*-6	22:5, *n*-3	22:6
						Neutral Lipid					
Peanut	304.0	62.0	33.7	516.2	148.6	6.3	29.4	0.8	6.4	0.1	3.1
Safflower	354.4	52.8	40.8	320.0	637.6	6.2	72.8	1.4	15.6	0.8	2.8
Soybean	449.3	77.7	44.4	497.2	580.4	47.0	47.6	6.2	2.9	8.4	16.6
Linseed	350.7	55.4	51.8	400.8	175.8	315.2	14.8	48.4		31.0	28.4
						Cholesterol Ester					
Peanut	10.8	2.7	1.6	19.4	5.6	0.2	4.7	0.1	0.2		0.6
Safflower	19.5	4.3	2.8	24.8	33.8	0.2	9.8	T	0.5		0.4
Soybean	65.2	12.4	7.2	94.4	78.3	4.8	10.5	0.5	0.2	0.6	1.5
Linseed	32.2	5.8	5.5	41.8	14.8	18.0	2.2	4.2		1.4	1.8
						Glycerides					
Peanut	243.2	46.0	25.2	433.4	116.9	5.6	12.6	1.1	3.1		0.4
Safflower	273.2	36.2	27.0	244.4	515.7	6.2	40.1	1.3	11.0	0.5	1.0
Soybean	296.6	48.2	25.8	330.8	409.5	32.4	21.8	3.4	1.5	5.8	11.2
Linseed	245.0	37.3	34.6	298.0	132.4	233.0	8.2	25.6	1.2	23.2	23.6
						Free Fatty Acids					
Peanut	73.0	16.2	12.6	109.8	40.4	2.0	16.0	0.3	2.8	T	0.7
Safflower	88.9	14.0	12.4	61.0	126.1	1.8	25.3	0.4	3.9	0.2	1.2
Soybean	94.4	19.2	13.6	93.6	109.8	11.8	17.5	2.6	0.6	2.4	5.9
Linseed	65.7	11.8	11.9	58.9	27.6	61.9	4.8	19.2		7.0	7.2
						Cephalin					
Peanut	86.6	9.0	148.0	87.1	90.9	2.4	189.5	1.1	50.8	0.6	19.4
Safflower	86.6	6.0	141.1	56.5	135.4	1.0	175.3	0.8	41.3	1.7	18.3
Soybean	90.4	7.1	140.1	65.0	121.3	3.4	165.3	2.8	3.8	9.8	65.0
Linseed	104.6	8.2	174.0	68.2	104.8	25.6	78.8	76.8	1.2	37.0	102.7
						Lecithin					
Peanut	230.9	14.0	243.5	116.8	107.9	4.0	338.1	2.0	51.4	0.7	15.2
Safflower	223.8	11.7	214.6	72.4	145.8	2.1	331.2	2.6	45.0	2.8	15.4
Soybean	224.1	13.3	209.0	75.8	150.1	2.5	288.3	5.6	5.0	7.8	53.4
Linseed	266.2	18.0	255.2	89.2	183.3	16.2	105.6	136.9		26.1	87.6

[a] Concentration is in mg of fatty acid/100 g of liver tissue and lipid fractions are from composite liver samples. In columns where T appears amount detected was less than 0.1 mg of fatty acid/100 g of tissue. [b] Eicosaenoic acid (20:1) is included in these values.

lipid class separations from the columns were monitored by thin layer chromatography on silica gel G using the following solvent systems for neutral lipids and phospholipids, respectively: hexane–diethyl ether–acetic acid (90:10:1, v/v/v) and chloroform–methanol–water (65:25:3, v/v/v).

Heneicosanoic acid was added as an internal standard to the lipid fractions, and methyl esters of the fatty acids were prepared as described by Worthington et al. (1972). Gas–liquid chromatographic determination of the fatty acid methyl esters was accomplished using butane-1,4-diol succinate or phosphate stabilized ethylene glycol succinate columns as described previously (Landes and Miller, 1974).

Total cholesterol determinations were made using a stable Liebermann-Burchard reagent (Kim and Goldberg, 1969). Measurements were made directly on serum samples and portions of the total lipid extracts of the heart samples, and on liver samples after saponification with alcoholic potassium hydroxide and extraction with light petroleum ether.

RESULTS AND DISCUSSION

Different vegetable oils were used in this study to give a wide range in dietary linoleic and linolenic acid content. However, one should keep in mind that there is considerable varietal variation in fatty acid composition within each of these oil seed species.

The fatty acid distributions of the dietary oils are given in Table I. Linolenic acid (18:3) ranged from trace amounts to 52% of the total fatty acids. However, linoleic acid (18:2) was not below 16% of the total fatty acids in any of the diets, and at this level makes up approximately 3.5% of the total energy of the diet, which is considerably more than was used in the work of Holman and Mohrhauer (1963).

The animals adapted to the experimental diets well, and a summary of the effects of the different oil treatments is given in Table II. The dietary oil treatments had no significant effect on the food intake, body weight, or organ and tissue weights. Neither the total cholesterol nor moisture content of the tissues analyzed was affected by dietary treatment. The total lipid concentration in the livers of the animals receiving peanut oil was reduced while that in the livers of the rats fed soybean oil was increased when compared to the animals receiving safflower and linseed oil. These differences were confined to the neutral fraction of the total lipid and thus could be related to some extent to absorption of fatty acids. The animals receiving the peanut oil may not have absorbed the very long chain fatty acids present in this oil very efficiently (Mattson and Streck, 1974).

Table III gives a summary of the concentration of selected fatty acids in the liver lipid fractions. The 16- and 18-carbon fatty acids make up 90% or more of the total fatty acids present in the neutral lipid. The dietary composition and the neutral lipid composition of this group of fatty acids are closely related with the possible exception

Table IV. Concentrations of Selected Fatty Acids in Heart Lipid Fractions of Rats Fed Different Vegetable Oils[a]

	16:0	16:1	18:0	18:1	18:2	18:3[b]	20:4	20:5	22:4, *n*-6	22:5, *n*-6	22:5, *n*-3	22:6
						Neutral Lipid						
Peanut	46.7	5.3	16.8	104.0	48.2	2.8	14.8	0.5	2.7		0.3	2.1
Safflower	42.6	3.7	16.8	42.7	118.7	0.9	14.1	0.5	3.2		0.2	1.0
Soybean	56.0		16.9	66.1	92.5	7.2	11.9	1.0	0.4		1.6	3.6
Linseed	38.3	4.2	16.2	54.4	31.6	48.7	6.1	3.6	0.1		4.1	4.5
						Cephalin						
Peanut	7.5	54.2	167.9	69.9	208.5	2.2	141.4		17.8	119.4		35.5
Safflower	9.5	52.0	177.6	37.8	261.7	0.9	129.0		29.4	123.9	T	33.2
Soybean	9.6	61.9	190.5	49.1	256.2	2.8	138.6		11.5	13.7	22.3	156.4
Linseed	9.1	53.0	197.8	52.5	178.4	23.7	91.2	16.7			50.4	156.3
						Lecithin						
Peanut	129.7		203.3	82.6	80.4	1.4	234.3		9.3	25.0	T	8.4
Safflower	125.0	1.6	198.8	45.9	106.1	0.7	215.7		17.2	28.8	1.7	7.9
Soybean	134.0	2.3	202.6	60.7	109.5	1.9	206.0		5.7	T	12.0	36.9
Linseed	120.6	4.3	210.7	72.0	40.9	15.3	105.3	17.6	T		35.6	46.2

[a] Concentrations are in mg of fatty acid/100 g of heart tissue. The lipid fractions are from pooled samples. In columns where T appears amount detected was less than 0.1 mg of fatty acid/100 g of tissue. [b] Eicosaenoic acid (20:1) is included in these values.

of the peanut oil treatment. The amount of palmitic (16:0) and linoleic acids was lower in the neutral lipid of this dietary group when compared to the other groups even though the diet contained more of these two acids than the linseed oil treatment. The presence of linolenic acid in more than trace amounts in the diet resulted in accumulation of the metabolites of this acid in the VLCPFA class. The animals receiving the linseed oil diet had a higher concentration of VLCPFA including metabolites of linoleic acid than any other group.

Data from Table II indicate that the increase in neutral lipid of the livers observed in the soybean oil fed animals occurred mainly in the cholesterol ester and glyceride fractions. This was especially evident when compared to the peanut oil fed group. When the fatty acids (Table III) of these two lipid fractions for these two treatments are examined, one sees that there was accumulation of palmitic, oleic (18:1), and linoleic acids in the cholesterol ester fraction of the soybean oil fed animals at the expense of the VLCPFA. However, this was not the case for the glyceride fraction. This fraction for the soybean oil treatment had a higher concentration of VLCPFA than for the peanut oil treatment with linoleic and palmitic acids being accumulated to some degree. There was a reduction in the 16- and 18-carbon fatty acids and accumulation of the VLCPFA for both the glyceride and free fatty acid fractions in the animals receiving the linseed oil diets.

There was little or no dietary effect on the phospholipid fractions of the liver with the exception that there may have been a slight elevation of these fractions in the animals receiving linseed oil (Table II). The VLCPFA concentrations of the liver phospholipid fractions (Table III) indicate that the reduction in the *n*-6 metabolites of the liver cephalin fraction for the soybean oil treatment was due mainly to disappearance of docosapentaenoic acid (22:5, *n*-6) while the reduction for the linseed oil treatment was due to a decrease in arachidonic acid (20:4). As the linoleic acid metabolites decreased, the metabolites of linolenic acid increased. The increase for the soybean oil treatment was confined mainly to increased accumulation of docosahexaenoic acid with some increase in docosapentaenoic acid (22:5, *n*-3). The large increase in linolenic metabolites for the linseed oil treatment occurred in all three VLCPFA of the *n*-3 series. As with the cephalin fraction, the linolenic metabolites were low in the liver lecithin for the peanut and safflower oil treatments with docosahexaenoic acid being most abundant, and the metabolites of linoleic acid remaining fairly constant. However, on the soybean oil treatment, the linoleic metabolites decreased with almost all of the docosapentaenoic acid (*n*-6) disappearing. This was accompanied by increases in the linolenic derived fatty acids. In the linseed oil treatment, no docosapentaenoic acid (*n*-6) was detected and the arachidonic acid was greatly reduced. A large increase occurred in the eicosapentaenoic acid along with other members of the linolenic metabolites; however, these increases did not offset the decreases in the acids derived from linoleic acid. This resulted in a reduction in the overall VLCPFA concentration in the lecithin fraction of the liver.

While the total lipid and the neutral lipid fraction of the heart tissue were not affected by dietary treatment (Table II), it is interesting that the ratio of cephalin to lecithin was altered. The animals that were fed peanut oil had a higher concentration of lecithin while the animals fed soybean or linseed oil had a higher concentration of cephalin. When the fatty acid composition of these fractions is considered (Table IV), one can see that for the cephalin fraction there was a large increase in the VLCPFA of the linolenic acid family in the animals receiving the soybean and linseed oils, without a corresponding decrease in arachidonic acid. This was also accompanied by an increase in stearic (18:0) and linolenic acids while the levels of oleic and linoleic acids were not affected to a large extent. The lecithin fraction of the heart tissue has a higher concentration of palmitic acid than the cephalin fraction and generally a lower concentration of VLCPFA. It is notable that the VLCPFA of the linolenic acid family were not accumulated extensively in this fraction from the animals receiving the soybean and linseed oil diets; however, there was a large reduction in the amount of arachidonic acid in the samples from the linseed oil fed animals. This resulted in an overall reduction in the concentration of VLCPFA in the samples from the animals receiving the soybean and linseed oil diets. These two factors are probably the reason for this alteration in the ratio of cephalin to lecithin.

This study reaffirms the work of Holman and Mohrhauer (1963) in that there is preferential elongation and desaturation of linolenic acid as compared to linoleic acid even when both of these acids exceed metabolic requirements; however, the preference is not as marked as on low fat diets. It appears that the presence of an excessive

amount of linolenic acid in the diet as was the case with the linseed oil diet causes a reduction in the amount of VLCPFA in the lecithin fraction of both liver and heart.

It was also observed that only arachidonic acid of the linoleic series and docosahexaenoic acid of the linolenic series were present in significant amounts when the dietary linoleic or linolenic acids, respectively, were at low levels. This seems to indicate that the other metabolites of the 18-carbon acids may serve to some degree as reserve supplies for these two very long chain polyenoic fatty acids when the dietary supply of the precursors is abundant.

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Utilization of Volatile Fatty Acids in Ruminants. V. Purification of Acetyl-Coenzyme A Synthetase from Mitochondria of Lactating Bovine Mammary Gland

Shahida Qureshi and Robert M. Cook*

A procedure for purification of acetyl-CoA synthetase from mitochondria of lactating bovine mammary gland is described. The purification method employs $(NH_4)_2SO_4$ fractionation, and three chromatographic steps using first DE-23 cellulose, DE-52 cellulose, and finally calcium phosphate. A 760-fold purification was achieved. The molecular weight is 63,000 as determined by sucrose density gradient centrifugation and the sedimentation coefficient is 4.4 S. Michaelis-Menten constants for Mg, CoA, ATP, and acetate are 6.51×10^{-4}, 2.92×10^{-4}, 2.24×10^{-4} and 6.10×10^{-4} *M*, respectively. Three bands of enzyme activity could be detected upon polyacrylamide gel electrophoresis. The data indicate that mammary acetyl-CoA synthetase exists as multiple forms.

Procedures for the purification of acetyl-CoA synthetase from bovine heart mitochondria have been reported by Webster (1965). When we used these procedures to attempt to purify the enzyme from bovine heart or mammary gland mitochondria, standard tests used to demonstrate purity (i.e., polyacrylamide electrophoresis and sedimentation equilibrium studies) indicated the presence of more than one protein in the purified preparations. Similarly, Farrar (1970) was unable to obtain a preparation of acetyl-CoA synthetase that showed a single protein band on polyacrylamide gel electrophoresis.

By employing purification procedures that are somewhat different from those reported by Webster (1965) and by using column chromatography on calcium phosphate gel as reported by Huang and Stumpf (1970), we have obtained acetyl-CoA synthetase in a highly purified form from the mitochondria of the lactating bovine mammary gland. Mammary tissue was removed in early lactation. The data indicate that acetyl-CoA synthetase exists in multiple forms. The details of these experiments are reported in this paper.

EXPERIMENTAL SECTION

Materials. Coenzyme A, acetyl-CoA, ATP, and AMP were purchased from Sigma Chemical Company. DEAE-cellulose (DE-23 and DE-52) was from Whatman. Calcium phosphate gel was prepared according to the method of Miller et al. (1965). Ovalbumin was obtained from Pharmacia. The chemicals for polyacrylamide gel electrophoresis were purchased from Canalco. Ammonium sulfate used throughout the experiment was a special enzyme research grade from General Biochemicals.

Enzyme Assay. Acetyl-CoA synthetase activity was assayed by measuring the acetate-dependent disappearance of the free sulfhydryl group of coenzyme A. The procedure was a slight modification of that reported by Mahler et al. (1953). In a total volume of 0.2 ml the complete reaction mixture contained 5 μmol of potassium acetate, 1.1 μmol of dipotassium ATP, 1.5 μmol of $MgCl_2$, 0.17 μmol of CoA-SH, 16 μmol of Tris-HCl (pH 8.6), and from 4 to 25 μg of protein. Blank tubes did not contain coenzyme A. The incubation time was for 3 or 10 min at 37°. The reaction was stopped by adding 2.8 ml of the nitroprusside reagent prepared according to the method of Grunert and

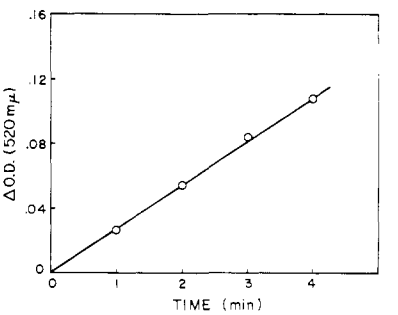

Figure 1. Effect of time on the linearity of the acetyl-CoA synthetase reaction.

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