Influence of diets containing different natural oils on the incorporation of [1-^{14}C]acetate in the various lipid fractions of rat liver

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1. A comparative study was undertaken with rats on the effect of various diets (normal stock, fat-free, palm oil and olive oil) on the in vitro incorporation of [^{14}C]acetate by the liver into cholesterol and into the fatty acids of phospholipids and neutral fats. 2. The total lipids extracted from the incubation mixtures were fractionated into acetone-precipitable and digitonin-precipitable portions and also into the fatty acids of neutral lipids. 3. The incorporation of [^{14}C]acetate into the acetone-precipitable fraction and into fatty acids of neutral fats was greatest in livers of rats given the fat-free diet, followed by those of the groups given olive oil, the normal stock diet, and palm oil. Livers from the group given the fat-free diet also exhibited the highest percentage of ^{14}C activity in the digitonin-precipitable fraction and were closely followed by the group on the normal stock diet. Compared with those of the other two groups, the livers of the groups given olive oil and palm oil showed much less activity in the digitonin-precipitable fraction. 4. The greater the amount of a specific type of fatty acid in the diet, the less was the ^{14}C activity incorporated into that type of fatty acid in the neutral fats of liver slices, but this was not so with the fatty acids obtained from the acetone-precipitable fraction of the lipids.

The fatty acid composition of the animal body is influenced by many factors including calorie intake, the quantity and quality of dietary fat and hormones (Haven & Bloor, 1956; Hegsted, Whyman, Gotsis & Andrews, 1960; Kline, McPherson, Pritchard & Rossiter, 1956*a*, *b*; Tischer, Opalka & Glenn, 1963). That the rate of fatty acid synthesis can itself be markedly altered by changes in the nutritional state was demonstrated by Boxer & Stetten (1944) and Bernhard & Steinhauser (1944). Both these groups of workers concluded that lipogenesis is greatly curtailed by restricted calorie intake.

The study now presented was undertaken to investigate the influence of various natural oils, given in the diet, on the incorporation of [1-^{14}C]acetate into the phospholipids, fatty acids and cholesterol of rat liver slices.

EXPERIMENTAL

Rats and diets

Two separate experiments were carried out, one with 25-day-old male weanling rats weighing 75–85 g and another with male rats 80–90 days old weighing 240–255 g, both of the Sprague–Dawley strain. In both, the animals were divided into four groups of seven each. They were caged individually and were maintained at 19·5–25°. One group of rats was given 10% olive oil by weight in a purified fat-free basal diet, another group was given 10% palm oil by weight in the same basal diet and a third group was

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maintained on the fat-free basal diet. The basal diet consisted of (by weight) 20 parts of casein (extracted twice with ethanol–water (1:1, v/v), once with ethanol–water (9:1, v/v) and once with diethyl ether), 60 parts of sucrose, 4 parts of mineral salts and one part of vitamin mixture. The salt mixture was identical with that described by Enser & Bartley (1962) and the vitamin mixture contained (mg/g): thiamine hydrochloride 21, riboflavine 7·9, nicotinamide 17, calcium pantothenate 28, pyridoxine hydrochloride 8, biotin 2, folic acid 3, *meso*-inositol 350, choline chloride 400, cyanocobalamin 0·1, menaphthone 3, *p*-aminobenzoic acid 160. Retinyl palmitate, ergocalciferol and α-tocopherol were dissolved in peroxide-free diethyl ether and sprinkled over the diet preparation providing (mg/kg diet): retinol 10, ergocalciferol 1·0, and α-tocopherol 100. The rats on the fat-free basal diet received 0·25 ml maize oil daily in their diet in order to ensure that there was no deficiency of essential fatty acids. A fourth group was maintained on a commercial rat diet. The diets were given to the rats for 30 days. Rats receiving the palm-oil diet were fed *ad lib.* and the rats of other groups were given a daily ration isocaloric with that eaten by the rats in the palm-oil group. All rats had free access to water, and coprophagy was prevented.

Incubation of tissues

The animals were killed by decapitation in the fed condition; the livers were removed and sliced and weighed amounts of the slices (0·5–1·0 g) were incubated with 5 μc [1-^{14}C]acetate in 5·0 ml Krebs–Ringer bicarbonate buffer (pH 7·4) that had been saturated with 95 % O_2 + 5 % CO_2 at 0°. The concentration of radioactive acetate in the Krebs–Ringer bicarbonate buffer was 0·003 mg/ml and the specific activity of the acetate used was 354 μc/mg which amounted to 1 μc/ml of the buffer. The incubation was carried out at 37·5 ± 0·5° in an atmosphere of 95 % O_2 + 5 % CO_2, for 3 h in a Dubnoff shaker. At the end of the incubation period the total lipids were extracted, separated into an acetone-precipitable fraction (phospholipids), a digitonin-precipitable fraction (cholesterol), and the fatty acids of the neutral lipids.

Extraction of lipids and methods of analysis

Total lipid extractions were carried out by a procedure similar to that of Folch, Lees & Stanley (1957). Tissues were homogenized in a Virtis-45 homogenizer for 3 min with chloroform–methanol (1:1, v/v), 20 volumes of the solvent mixture/g tissue being used. The homogenate was warmed to 40–45° for 15 min while being stirred in an atmosphere of nitrogen. It was centrifuged and the clear supernatant liquid was filtered through a fat-free filter paper. The residue was re-extracted with 20 volumes of chloroform–methanol mixtures (2:1 and 3:1, v/v) and finally with diethyl ether. The total extracts were combined and the solvents were removed in a rotary film evaporator at 40–45°.

The total lipids were re-extracted with three 5 ml portions of redistilled hexane and the volume of combined extracts was reduced to 2 ml by evaporating in a current of nitrogen. To each volume of hexane extract were added twenty volumes of redistilled acetone and 0·3 volumes of 30 % (w/v) $MgCl_2$ in 95 % (v/v) ethyl alcohol and the mixture was stirred for 2 min. The precipitate was centrifuged and the supernatant

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fraction was preserved for the isolation of cholesterol and fatty acids. The precipitate was redissolved in a minimum volume of hexane and reprecipitated with acetone as before. The final precipitate, representing the total phospholipids, was dissolved in 10 ml chloroform, and 1 ml of the solution was transferred to a clean planchet, the solvent removed with an infrared lamp and the radioactivity determined (Chase & Rabinowitz, 1963) using a windowless gas flow counter (Tracerlab, Inc., Waltham, Mass., USA).

A further portion (5 ml) of the phospholipid solution was taken and the solvent was removed in a stream of nitrogen. The dry residue was dissolved in 15 ml methanol and 5 ml 4 N-HCl were added. The phospholipids were hydrolysed on a steam bath for 1–2 h, by the method of Dawson, Hemington & Davenport (1962). After the hydrolysate had been cooled, the liberated fatty acids were extracted with three 50 ml portions of redistilled light petroleum (b.p. 40–60°). The extracts were combined, washed three to five times with distilled water, dried over anhydrous sodium sulphate, and the solvent was evaporated in a current of nitrogen. The free fatty acids thus obtained were dissolved in a minimum quantity of peroxide-free diethyl ether and a three- to five-fold excess of freshly prepared diazomethane was added at 0° in order to prepare the methyl esters of the free fatty acids (De Boer & Backer, 1956; Schlenk & Getterman, 1960). The mixture was allowed to stand for 30 min, the excess diazomethane and diethyl ether were evaporated in a current of nitrogen the methyl esters of the fatty acids were dissolved in 10 ml redistilled light petroleum (b.p. 40–60°) and a portion of 1 ml was taken for determination of radioactivity. The remaining methyl esters were separated into saturated, mono-, di- and poly-unsaturated esters by a method similar to that of De Vries (1963). The fatty acids were analysed by gas–liquid chromatography with a Beckman GC-2A analytical instrument fitted with a hydrogen-flame ionization detector. The stationary phase was 15% (w/w) diethyl glycol succinate on acid-washed chromasorb W (60–80 mesh). Analyses were made at a column temperature of 195° and at a gas flow rate of 60 ml/min.

The acetone-soluble fraction of the lipids remaining after the precipitation of phospholipids was evaporated to dryness and re-extracted with ethyl alcohol. The ethanol extract was saponified by adding 2 ml 50% (w/v) KOH and refluxing on a steam bath for 3–4 h by the method of Burchfield & Stores (1962). The contents were diluted with distilled water, and the non-saponifiable portion (cholesterol) was extracted twice with 75 ml portions of light petroleum. The total extracts were pooled, washed free of alkali with distilled water, dried over anhydrous sodium sulphate, and the solvent was evaporated to dryness in a current of nitrogen. The residue was re-extracted three times with 2 ml portions of ethyl alcohol–acetone (1:1, v/v). The extract was acidified with one drop of N-acetic acid, and cholesterol was precipitated as the digitonide by the method of Sperry & Webb (1950). The cholesterol digitonide was washed first with diethyl ether–acetone (3:1, v/v) and then with diethyl ether. The pure digitonide was dissolved in methanol and a portion of the solution was taken for determination of radioactivity.

The contents remaining after the extraction of the non-saponifiable matter were freed completely of ethanol by warming in a steam bath in a current of nitrogen. The

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cooled contents were acidified with 10 N-sulphuric acid and the free fatty acids were extracted three times with 50 ml portions of petroleum ether. The combined extracts were washed several times with distilled water to remove acid, dried over anhydrous sodium sulphate, the solvent was evaporated to the required volume and a portion taken for determination of radioactivity. The remaining free fatty acids were converted into their methyl esters and the methyl esters were separated into saturated, mono-, di-, and poly-unsaturated esters as described above.

The radioactivity of fatty acids, phospholipids and cholesterol was determined in a windowless gas flow counter as infinitely thin film preparations and activities thus obtained were corrected for self-absorption.

RESULTS AND DISCUSSION

The fatty acid compositions of the dietary oils (olive oil, palm oil and maize oil) used to supplement the basal fat-free diet are given in Table 1.

Table 1. *Fatty acid composition of the dietary oils expressed as a percentage of the total fatty acids*

Fatty acid	Palm oil	Olive oil	Maize oil
Myristic	1·3		
Palmitic	53·2	12·9	12·9
Palmitoleic	3·3	1·5	
Stearic	34·1	1·7	1·9
Oleic	8·1	77·0	29·8
Linoleic		6·9	55·4

Table 2. *Mean weight changes (g) of seven rats receiving one of four diets*

	Weanling rats			Adult rats		
Diet	Wt at the beginning* (g)	Wt at the end* (g)	Live-weight gain (g/day)	Wt at the beginning* (g)	Wt at the end* (g)	Live-weight gain (g/day)
10% palm oil	78 ± 6	178 ± 6	3·8	249 ± 8	331 ± 13	2·7
10% olive oil	81 ± 4	198 ± 8	4·5	244 ± 9	348 ± 11	3·4
Fat-free	82 ± 2	166 ± 5	3·2	248 ± 8	278 ± 11	1·0
Normal	75 ± 4	202 ± 10	4·9	246 ± 7	352 ± 9	3·5

* Mean values and standard deviations.

The mean growth rates of different groups of rats (Table 2) show that the maximum growth rate occurred in the weanling rats which received the normal stock diet. The olive oil used for the feeding experiments contained 7% linoleic acid and 77% oleic acid, whereas the palm oil contained no linoleic acid and only 8% oleic acid; this suggests that the diet with palm oil was deficient in essential fatty acids. The decrease in the growth rate of the group of rats given the palm oil supplement may thus be attributed to a deficiency of essential fatty acids. The growth rate of the groups of rats maintained on a fat-free diet supplemented with maize oil was the lowest, in spite of the fact that they received just sufficient linoleic acid in their diet. The results with 3-month-old rats were similar, except that the rats that received the fat-free basal

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diet showed a still lower growth rate than the rats kept on diets supplemented with palm or olive oil.

The maximum incorporation of radioactivity into the acetone-precipitable fraction and the free fatty acids of the neutral lipids occurred in the liver tissues of the group of rats that received the fat-free diet supplemented only with 0·25 ml maize oil per day (Table 3). The groups of rats maintained on the olive oil diet, normal stock diet

Table 3. *Labelling of phospholipids, fatty acids and cholesterol from [1-^{14}C]acetate by liver tissues from seven rats receiving one of four diets*

(Counts/g wet tissue; mean values and standard deviations)

	Weanling rats			Adult rats		
Diet	Phospholipids	Fatty acids	Cholesterol	Phospholipids	Fatty acids	Cholesterol
10% palm oil	18618 ± 1238	19870 ± 1627	5386 ± 209	41829 ± 2072	25133 ± 2371	5647 ± 981
10% olive oil	43338 ± 1432	40906 ± 1290	4748 ± 187	64277 ± 3401	45949 ± 3401	4982 ± 870
Fat-free	71932 ± 3845	74752 ± 1790	12674 ± 641	78268 ± 3485	56852 ± 2343	21878 ± 2011
Normal	28960 ± 655	25788 ± 1366	10082 ± 517	31452 ± 1212	34388 ± 892	17380 ± 780

Table 4. *Distribution of ^{14}C activity (expressed as a percentage of the total radioactivity) in the group of fatty acids in the neutral lipids obtained from the livers of seven rats receiving one of four diets*

	Weanling rats				Adult rats			
Diet	Saturated	Monounsaturated	Diunsaturated	Polyunsaturated	Saturated	Monounsaturated	Diunsaturated	Polyunsaturated
10% palm oil	48·8	28·0	5·6	17·7	54·9	18·6	2·0	24·5
10% olive oil	72·9	11·8	2·0	13·4	73·9	16·3	1·7	8·1
Fat-free	51·1	34·7	2·9	11·3	59·2	28·3	1·2	11·3
Normal	46·6	28·0	6·0	19·4	51·9	24·1	2·0	22·0

or palm oil diet incorporated radioactivity into the acetone-precipitable fractions and free fatty acids of neutral lipids in descending order. Incorporation of radioactivity into the digitonin-precipitable fraction of the lipids was found to be slightly higher in rats given the palm oil supplement than in those given the olive oil supplement, but was much lower than in those on the basal diet. With weanling rats, however, the group given the fat-free diet incorporated more than twice as much of [1-^{14}C]acetate into this fraction than the groups given palm oil or olive oil supplements; in the 3-month-old rats receiving the fat-free diet the activity in this fraction was about four times as high as in those receiving palm oil or olive oil. The radioactivity of digitonin-precipitable fraction from the rats kept on the normal stock diet was almost the same as that of the fat-free group, whereas the radioactivity of fatty acids of neutral fats and that of phospholipids of the group given normal stock diet was very much lower than that of the groups of rats kept on the fat-free or olive oil diets even though the total fat content of the normal stock diet was about 8% by weight.

The total fatty acids in the diet supplemented with palm oil contained about 89% saturated and 11% monounsaturated, but no polyunsaturated fatty acids. The livers of rats receiving this diet incorporated [1-^{14}C]acetate to give 49, 28 and 23% radio-

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activity in the saturated, mono- and poly-unsaturated fatty acids respectively (Table 4). The total fatty acids in the diet supplemented with olive oil contained about 15% saturated 78% monounsaturated and 7% polyunsaturated fatty acids and in this group the percentages of radioactivity incorporated into the liver lipids were 73, 12 and 15 in the saturated, monounsaturated and polyunsaturated fatty acids respectively. The group of rats kept on the fat-free diet received only about 100 mg polyunsaturated, 60 mg monounsaturated and 30 mg saturated fatty acids per day. In this group the percentages incorporated were 51, 35 and 14 in saturated, mono- and polyunsaturated fatty acids respectively.

The pattern of acetate incorporation into the fatty acids of liver phospholipids was different from that of the fatty acids of neutral fats (Table 5). In the group given palm oil 88, 4 and 8% radioactivity occurred in saturated, mono- and poly-unsaturated fatty acids respectively. In the group given 10% olive oil the values were 77, 9 and 14% and in the group given the fat-free diet 74, 12 and 14% radioactivity in the saturated, mono- and poly-unsaturated fatty acids respectively. A close parallel relationship, in the percentage of [^{14}C]acetate incorporated into the saturated, mono- and poly-unsaturated fatty acids of liver, among the various groups of young and adult rats was observed in all the experiments.

Table 5. *Distribution of ^{14}C activity (expressed as a percentage of the total radioactivity) in the groups of fatty acids in the acetone-precipitable lipids obtained from the livers of seven rats receiving one of four diets*

	Weanling rats				Adult rats			
Diet	Saturated	Monounsaturated	Diunsaturated	Polyunsaturated	Saturated	Monounsaturated	Diunsaturated	Polyunsaturated
10% palm oil	87·7	3·8	3·0	5·5	83·8	6·8	2·3	7·1
10% olive oil	77·3	9·1	4·2	9·4	78·5	5·8	5·6	10·2
Fat-free	73·9	11·7	4·3	10·2	78·6	6·9	3·0	11·5
Normal	79·8	6·8	6·0	7·7	71·8	9·7	8·0	10·5

Whitney & Roberts (1955) have reported that liver slices from rats fed on a high-fat diet for 2–3 months exhibit a depressed capacity to incorporate [2-^{14}C]acetate into fatty acids and Brice, Okey & Stone (1956) state that the rats fed on a high-fat diet have a lower capacity to convert [2-^{14}C]acetate administered intraperitoneally into fatty acids. The work of Hill, Linazasoro, Chevallier & Chaikoff (1958) has also established the exquisite sensitivity of hepatic lipogenesis to fat ingestion. A review of the results in Tables 1, 4 and 5 reveals that, the higher the amount of a specific type of fatty acid in the diet, the lower was the incorporation of [1-^{14}C]acetate into that type of fatty acid. This is consistent with the major homoeostatic concept of lipogenesis (Masoro, 1962). In the results reported here the fat-free diet gave rise to increased incorporation of [1-^{14}C]acetate into the phospholipid, fatty acids and cholesterol fractions of the livers as compared to the incorporation of ^{14}C activity by the livers of rats given 10% olive oil or 10% palm oil in their diet.

With both the weanling and adult rats it can be observed from the results that rats kept on the diet supplemented with olive oil incorporated more [1-^{14}C]acetate into the

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saturated fatty acids of the neutral fats and less into mono- and poly-unsaturated fatty acids compared to those that received the diet with the palm oil supplement. It is of interest to note that the groups of rats that received the diet supplemented with palm oil and the normal basal diet had almost similar rates of incorporation of ^{14}C activity into saturated, mono- and poly-unsaturated fatty acids of neutral fats whereas in those rats that received the fat-free diet there was less ^{14}C activity, mainly in the poly-unsaturated fatty acids.

There was no significant difference in the incorporation of radioactivity into corresponding fatty acids of the liver phospholipids in the groups of rats given different oils in their diets. However, in weanling and adult rats, there was in those that received 10% palm oil a higher incorporation of radioactivity, from phospholipids, in the saturated fatty acids and a lower incorporation in the polyunsaturated fatty acids, a finding for which there is no explanation. The studies of Tischer *et al.* (1963) show that in rats kept on a fat-free diet, there is a decrease in linoleic acid to trace levels in all phosphotides except cardiolipin, where, also, the level fell from 80 to 8% along with rapid reduction of arachidonic acid in all phospholipids except in phosphatidyl ethanolamine. In the experiments presented now, the rats kept on the fat-free diet had ample essential fatty acids in their diet. The main polyunsaturated fatty acids recognized by gas–liquid chromatography in these experiments were linoleic and arachidonic acids.

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