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ARACHIDONIC ACID AND METHODS FOR ITS ISOLATION FROM NATURAL MATERIALS

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Methods that have been developed for isolating arachidonic or, in full, cis-eicosa-5,8,11,14-tetraenoic, acid from the wastes of the endocrine industry are described. The acid isolated has been characterized by physicochemical and spectral features. Prostaglandin E_2 has been obtained by enzymatic synthesis from arachidonic acid.

Arachidonic acid, together with other polyunsaturated acids, is a structural component of the lipoproteins of cell membranes and participates in the performance of a number of very important biochemical processes in the cell, which ensure the vital activity of the organism. The rising interest in arachidonic acid and methods for its isolation in recent times is due to the transformations of this acid into a whole series of biologically important metabolites — prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), etc. The processes for forming these metabolites in the living cell are based on the enzymatic oxidation of arachidonic acid with subsequent biotransformation into the final compounds.

Thromboxanes are closely interlinked with the processes of thromogenesis and hemopoiesis, and the leukotrienes participate in allergic (anaphylactic) reactions of the organisms. The prostaglandins, which are known as intracellular bioregulators of many physiologically important processes, exert an influence on the cardiovascular, respiratory, reproductive, and other systems.

The prostaglandins, thromboxanes, and leukotrienes are finding wide use in human and veterinary medicine. Already today, the use of prostaglandins has been described for the treatment of hypertonia, bronchial asthma, vascular thrombosis, and gastric ulcers, and in gynecology, etc. [1].

One of the promising methods of obtaining prostaglandins E_2 and $F_{2\alpha}$, thromboxanes A_2 and B_2, and prostacyclin I_2 is their enzymatic synthesis from arachidonic acid with the aid of specific multienzyme complexes isolated from various sources of animal origin.

Arachidonic acid (Δ^4Ach) is widely distributed in animal tissues. However, its isolation is complicated by its high sensitivity to oxidation and by its low percentage in the materials mentioned, and also by the fact that it is present together with other polyenoic acids with similar physicochemical properties. Arachidonic acid in the form of its methyl ester with different degrees of purity (87-96%) has been obtained from cattle adrenal glands [2, 3] and from porcine liver [4].

In the present paper we describe methods which we have developed [5] for isolating arachidonic acid from the lipids of the cattle pancreas, which are wastes of the endocrine industry. The proposed methods for isolating arachidonic acid from lipid wastes of the currently existing manufacture of endocrine preparations makes it possible to have not only the desired product but also to create a wastefree production for a number of preparations of the endocrine industry.

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The isolation of arachidonic acid is carried out through the stage of obtaining concentrates: fatty acids (CFA, 4.5% Δ^4Ach), unsaturated fatty acids (CUFA, 22.9% Δ^4Ach), arachidonic acid methyl esters (CME $\Delta^4Ach_{a,b,c}$), and methyl arachidonate (ME Δ^4Ach) with its subsequent saponification to arachidonic acid (scheme).

The saponification of the lipids with aqueous alcoholic alkali, acidification, and extraction with toluene gave the CFA. The amount of arachidonic acid in the fatty acid concentrate obtained (Table 1) was 4.52%. The main impurities were palmitic acid (22.9%) and oleic acid (45.0%). These saturated and monoenoic acids were separated on the basis of the different boiling points of their esters, different solubilities of the fatty acids in acetone, and their inclusion compounds with urea in methanol at low temperatures.

Acid fraction O_1 (scheme, Table 1), containing mainly palmitic and stearic acids, was separated by crystallization from acetone at −30°C and at fraction O_2, containing mainly oleic acid, at −70°C. Thus, the CUFA (Δ^4Ach 22.9%) containing oleic acid was obtained practically without loss of arachidonic acid. A concentrate of methyl arachidonate (MeE Δ^4Ach) was obtained in three ways (scheme and Table 1):

a) Methyl oleate was eliminated by fractional distillation of the methyl esters of the CUFA with the fractions F_1 and F_2 and a 43.5% CME Δ^4Ach_a was obtained (with a 72.6% yield in terms of arachidonic acid);

b) by crystallization at 20°C of the inclusion complexes with urea of the CUFA, the oleic acid was eliminated with fraction O_3, giving, after methanolysis, a 42% CME Δ^4Ach_b (with a 78.5% yield in terms of the arachidonic acid); and

c) by crystallization the inclusion complexes of the CUFA with urea the oleic acid (O_3 fraction) was separated at 20°C. At −50°C, the separation of oleci acid took place and also, to a greater extent than at 20°C, that of linoleic, linolenic, eicosatrienoic, and pentaenoic acids. The CME Δ^4Ach_c obtained in this way has a high concentration of Δ^4Ach (54.5%) in comparison with CME $\Delta^4Ach_{a,b}$ and a lower concentration of trienoic and pentaenoic acids, which is extremely important in the isolation of high-percentage concentrates of ME Δ^4Ach_b. The yield of arachidonic acid in method c was 65.3%.

The fractions separated consisting of the wastes of various methods of obtaining CME Δ^4Ach (F_1, F_2, O_3, and O_4, containing 4.6, 15.0, 8.4, and 20.5% of Δ^4Ach, respectively) — after their conversion into acids — can be returned to the process at the stage of the crystallization of the CFA from acetone at −70°C.

The isolation of methyl arachidonate (ME Δ^4Ach) from the CME $\Delta^4Ach_{a,b,c}$ obtained was carried out either by adsorption chromatography on argentized silica gel (variant A, scheme) or by rectification followed by chromatography (variants B and C). Thus, the chromatographic purification of a 42% CME Δ^4Ach_b on argentized silica gel gave a 99.2% methyl arachidonate with a yield of 60% (Table 2, variant A). The fractionation of the CME Δ^4Ach_b in a vacuum of greater than 0.05 mm in a fractionating column with a reflux number of 15 permitted the isolation of a 93.5% ME Δ^4Ach_b the impurities in which (methyl eicosatrienoate (4.3%), the methyl ester of a tetraenoic acid isomeric with arachidonic acid (1.0%), and the methyl ester of a pentaenoic acid (1.2%)) were eliminated almost completely after purification on argentized silica gel (Table 2). By a similar fractionation, the CME Δ^4Ach_c containing smaller amounts of the trienoic acid, the isomeric tetraenoic acid, and the pentaenoic acid as impurities, as compared with CME $\Delta^4Ach_{a,b}$ (see Table 1) yielded a 97% ME Δ^4Ach_c, the column chromatography of which gave a 99.9% methylarachidonate.

The saponification of the 99.9% methyl arachidonate gave chemically pure arachidonic acid. The absence from the UV spectrum of the sample of absorption maxima for conjugated double bonds confirmed the stability of the arachidonic acid in the proposed methods for its isolation. The acid isolated was characterized by its physicochemical and spectral properties.

The samples of arachidonic acid obtained were used as substrates of the prostaglandin synthetase of the microsomes of ram vesicular glands for the enzymatic synthesis of PG E_2.

EXPERIMENTAL

TLC was performed on Silufol UV-254 (Czechoslovakia), using the petroleum ether—ethyl ether (6:4) system for the fatty acids and their esters and the benzene—dioxane—acetic acid (20:20:1) system for prostaglandin. The substances were detected on the chromatograms by

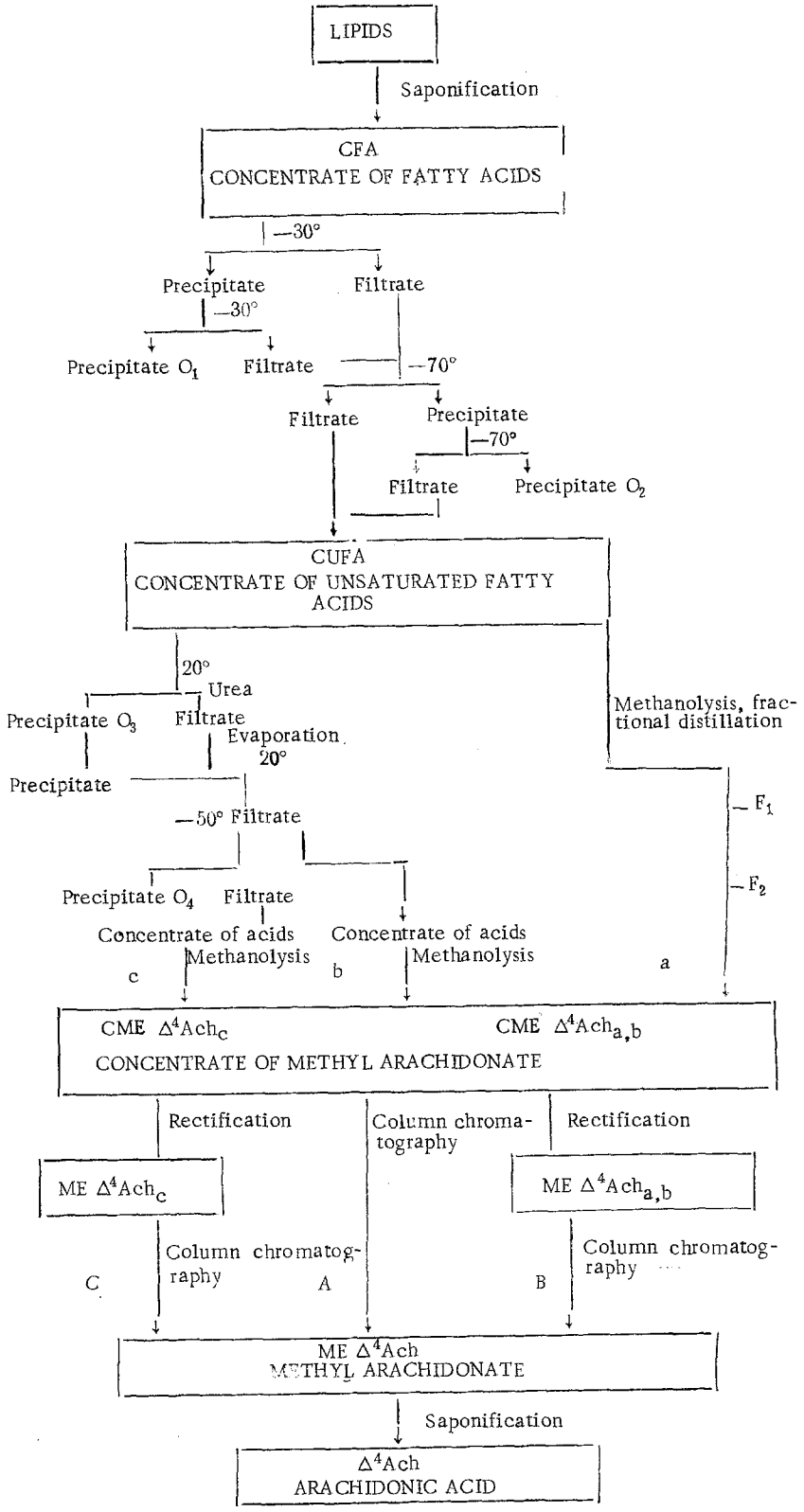
TABLE 1. Fatty Acid Compositions (%) of the Fractions in the Production of Concentrates of Methyl Arachidonate

Acid	CFA*	O_1	O_2	CUFA	Φ_1	Φ_2	O_3	O_4	CME · Δ^4Ach$_a$	CME · Δ^4Ach$_b$	CME · Δ^4Ach$_c$
1. Myristic and other lower acids	1,4	3,1	0,8	0,5	1,7		0,9				
2. Palmitic (16:0)	22,9	58,6	8,4	6,4	20,4	3,2	7,7	2,7		1,0	
3. Palmitoleic (16:1)	2,7	2,0	4,2	0,5	2,2		Tr.	4,5		1,6	
4. Palmitolinoleic (16:2)	1,3	2,2	1,2	0,2	0,6		0,5				
5. Palmitolinolenic (16:3)	1,1	0,7	2,8	Tr.			Tr.				
6. Stearic (18:0)	8,9	21,5	2,1	10,8	10,5	14,9	16 0	4,1	7,2	2,1	0,9
7. Oleic (18:1)	45,0	8,2	73,2	41,7	54,8	52,1	60,5	24,8	26,0	16,1	12,6
8. Linoleic (18:2)	6,1	3,7	7,3	7,0	4 5	7,7	3,3	17,0	8,0	12,9	10 6
9. Linolenoic (18:3)	5,7			2,9	0,6	2,5	0,7	8,9	4,2	6,5	5,9
10. Eicosenoic (20:1)	Tr.			Tr.			0,3				
11. Eicosadienoic (20:2)	Tr.			Tr.			0,5				
12. Eicosatrienoic (20:3)	0,42			2,2	0,3	1,0	0,7	6,8	3,5	4,3	2,9
13. Arachidonic (20:4)	4,52			22,9	4,6	15,0	8,4	20,5	43,5	42,0	54,5
14. Isomer of arachidonic	Tr.			Tr.			Tr.	0,4	Tr.	1,2	
15. Eicosapentaenoic (20:5)	0,96			4,9	0,8	3,6	0,5	10,9	7,6	12,3	11,6
Yield of the fraction on the fatty acids, %		32,2	47,8	20,0	5,3	3,6	11,3	3,2	8,0	8,5	5,2
Yield of arachidonic acid, %		0,0	0,0	99,7	5,3	11,7	20,8	14,0	76,2	78,5	65,3

*For the meanings of the abbreviations, see text.

TABLE 2. Fatty Acid Compositions (%) of the Fractions in the Production of Methyl Arachidonate (ME Δ^4Ach)

Acid	Variant A: fractions obtained on column chromatography, CME Δ^4Ach_b					Variant B: rectification of the CME Δ^4Ach_b				Variant B: column chromatography of the CME Δ^4Ach_b			Variant C: rectification of the CME Δ^4Ach_c				Variant C: column chromatography of the ME Δ^4Ach_c		
	I	II	III	IV	V	I	II	III	IV	I	II	III	I	II	III	IV	I	II	III
1. 16:0	4,0					3,8													
2. 16:1	6,3					5,7													
3. 18:0	8,3					7.3							5,3						
4. 18:1	66,6	0,5				52,1	13,2						58,0	12,0					
5. 18:2	12,9	46,4				22,6	31,7						27.5	34,2					
6. 18:3	1,9	29,0				8,5	22,9						9,2	23,6					
7. 20:3	Tr.	19.3	0,6				11.7	4,3		45,5	0,5			12,0	2,2		3,8	0,1	
8. 20:4		4,8	99,2	70,0			19,8	93,5	13,5	50,5	99,1			18,0	97,0	11,0	6,2	99,9	Tr.
9. 20:4 isomer			0,2	26,4	Tr.		0.7	1,0	Tr.	4,0	0,4	27,0							
10. 20:5				3,6	100,0		Tr.	1,2	86,5			73,0			0,8	89,0			99,9
Conditions: % ether in hexane or b.p.	4 0	15,0	50,0	50,0	75,0	162—176	177—189	190—195	196—199	15—50	50	50—75					15—50	50	50—75
Yield of the fraction on the CFA, %	2,04	1,66	2,70	0,36	1,07	2,00	1,50	2,80	1,00	0,22	2,40	0,04	0,83	0,75	2,38	0,65	0,13	2,10	0,01
Yield of arachidonic acid, %	0,0	3,3	60,0	4.7	0.0	0,0	6,7	58,0	3,0	2,5	52,7	0,0	0,0	3,1	51,0	1,5	1,7	48,5	0,0



Scheme of the isolation of arachidonic acid

spraying them with a 2% ethanolic solution of molybdophosphoric acid followed by heating at 100-120°C. Silica gel L 100/160 was used for column chromatography.

GLC was performed on Chrom-4 chromatograph (Czechoslovakia) fitted with a steel column (200 cm × 2 mm) filled with 10% of poly(ethylene adipate) on Chromosorb W-AW-DMCS at a temperature of 200°C with the carrier gas (helium) at the rate of 30 ml/min.

IR spectra were recorded on a Perkin-Elmer 257 instrument using films of the substances, PMR spectra on a Bruker WP-60 spectrometer (GFR) in $CDCl_3$ with tetramethylsilane as internal standard, and UV spectra on a Hitachi EPS-3T instrument. Mass spectra were recorded on a LKB-2091 chromato-mass spectrometer at an energy of the ionizing electrons of 22.5 eV with direct introduction into the ion source at 100°C for prostaglandin E_2.

Concentrate of Unsaturated Fatty Acids (CUFA). An emulsion of 400 g of a concentrate of the lipids from the wastes of endocrine production in 400 ml of ethanol was treated with 180 ml of a 40% aqueous solution of caustic potash. The reaction mixture was stirred at 40-45°C in a current of nitrogen for 3.5 h and was then cooled to 18-22°C, 200 ml of toluene was added, and the mixture was acidified with 150 ml of 60% sulfuric acid to pH 2-3. The precipitate that deposited was filtered off and washed with 100 ml of toluene. The organic layer of the filtrate was separated from the aqueous layer, and the aqueous layer was extracted with toluene (2 × 50 ml). The solvent was distilled off from the combined toluene extracts after they had been treated with a saturated aqueous solution of sodium metabisulfite, giving 177.0 g of a concentrate of fatty acids (CFA, Δ^4Ach 4.52%). A solution of 177.0 g of the fatty acids in 1200 ml of acetone was stirred at −30°C for 2 h. The precipitate that deposited was separated and was again stirred with a sevenfold volume of acetone at −30°C for 2 h. The deposit (O_1) (57.0 g) was separated off and the filtrates were combined and subjected to fractional separation at −70°C. The acetone was driven off from the final filtrate, giving 35.3 g of a concentrate of unsaturated fatty acids (CUFA, Δ^4Ach 22.9%).

Concentrate of Methyl Arachidonate (CME Δ^4Ach). a) A mixture of 35.3 g of CUFA (Δ^4Ach 22.9%) in 350 ml of anhydrous methanol and 3.5 g of acetyl chloride was stirred at 50-55°C for 1 h. The excess of methanol was eliminated, and the reaction mixture was neutralized with saturated sodium bicarbonate solution. The substance was extracted with toluene. After the solvent had been distilled off, the residue (33.2 g) was distilled at a residual pressure of 0.05 mm Hg. This gave three fractions: I (F_1) — 9.3 g, bp 110-124°C; II (F_2) — 6.3 g, bp 124-140°C; and III (CME Δ^4Ach) — 14.2 g, bp 141-180°C — a concentrate of methyl arachidonate (Δ^4Ach 43.5%).

b) 35.3 g of CUFA was added to a solution of 142.0 g of urea in 400 ml of methanol that had been heated to 60-65°C, and the mixture was stirred for 20 min and was left for 10 h. The solid matter was separated off and the filtrate was evaporated to half volume and the new precipitate that has deposited was combined with the preceding one (O_3). The resulting filtrate was acidified with 30 ml of 10% hydrochloric acid to pH 2-3 and was then stirred in a current of nitrogen at 40-45°C for 45 min, cooled, and treated with toluene (3 × 50 ml). The organic layers were separated off and the solvent was evaporated off. The residue (16.1 g) was stirred with 160 ml of anhydrous methanol and 1.6 g of acetyl chloride at 50-55°C for 1 h. After working up, 15.0 g of a concentrate of methyl arachidonate (CME Δ^4Ach_b, 42.0%) was obtained.

c) 33.5 g of CUFA was added to a solution of 142.0 g of urea in 400 ml of methanol that has been heated to 60-65°C and the mixture was stirred for 20 min and left for 10 h. The precipitate was separated off, the filtrate was evaporated to half bulk and the new precipitate that had deposited was combined with the preceding one (O_3). Then the filtrate was stirred at −(50-45°C) for 2 h. The precipitate (O_4) was filtered off and the filtrate was acidified with 28 ml of 10% acid to pH 2-3, and, after working up, 9.4 g of an arachidonic acid concentrate was obtained. A solution of 9.4 g of the concentrate in 100 ml of anhydrous methanol together with 1.0 g of acetyl chloride was stirred at 50-55°C for 1 h. After working up, 8.9 g of a concentrate of methyl arachidonate with CME Δ^4Ach_c 54.5%) was obtained.

Methyl Arachidonate (ME Δ^4Ach). A) A solution of 3.0 g of the concentrate of methyl arachidonate (CME Δ^4Ach_b, 42.0%) in 10 ml of a mixture of hexane and diethyl ether (96:4) was transferred to a column containing 90 g of silica gel (L 100/160) impregnated with $AgNO_3$ (10%) that had previously been washed with 50 ml of hexane—diethyl ether (96:4). The products were eluted with hexane—diethyl ether mixtures (containing 4, 15, 50, and 75% of ether in hexane), fractions with a volume of 5-6 ml being collected. The process was monitored by TLC on argentized silica gel. The eluates, containing mainly the methyl esters of saturated and monounsaturated acids, were combined to form fraction I, and the esters of dienoic and trienoic acids to form fraction II, while fraction III consisted of the ester of arachidonic acid, fraction IV the ester of arachidonic acid and an isomer of it, and fraction V the ester of a pentaenoic acid. The solvent was distilled off from fraction III, giving 1.01 g of methyl arachidonate with a purity of 99.2%.

B. The concentrate of methyl arachidonate CME Δ^4Ach (42.0%) (11.2 g) was fractionated at 0.05 mm Hg in a fractionating column (1.0 × 18 cm) with a glass packing at a reflex number of 15. From 10 to 15 fractions were collected and they were monitored by TLC on argentized silica gel. Fractions containing mainly methyl esters of saturated and monoenoic acids were combined into fraction I, those of dienoic and trienoic acids into fraction II, those of tetraenoic acids into fraction III, and those of pentaenoic acids into fraction IV. The third fraction (4.0 g) contained 93.5% of Δ^4Ach. A solution of 2.25 g of the methyl arachidonate obtained in 10 ml of 15% diethyl ether in hexane was transferred to a column containing 50 g of argenized silica gel and this was washed with 30 ml of diethyl ether in hexane (15%). The products were eluted with mixtures of diethyl ether in hexane (in concentrations of 15, 20, 50, and 75%), fractions with a volume of 5-6 ml being collected. The process was monitored by the GLC method. Fractions containing not less than 99.0% of Δ^4Ach were combined (fraction II, see Table 2). The solvent was driven off. This gave 2.14 g of methyl arachidonate with a purity of 99.1%.

C. The concentrate of methyl arachidonate CME Δ^4Ach (54.5%) (9.4 g) was separated in a fractionating column. This gave 4.2 g of methyl arachidonate with a purity of 97.0%. When 2.1 g of the ester obtained was purified by adsorption chromatography on argentized silica gel, 1.75 g of methyl arachidonate with a purity of 99.9% was obtained.

Arachidonic Acid (Δ^4Ach). Methyl arachidonate (99.9%) (1.75 g) in 45 ml of a mixture of a 5% aqueous solution of caustic soda and ethanol (1:2) was stirred in a current of nitrogen at 30-35°C for 3 h and was then cooled and acidified with 2 N sulfuric acid to pH 2. The substance was extracted with toluene, and the extract was washed with water to neutrality and with saturated sodium metabisulfate. Then it was dried with sodium sulfate and the solvent was driven off. After purification on silica gel, 1.5 g of arachidonic acid was obtained: d_4^{20} 0.9245, n_D^{20} 1.4862 (according to the literature [6]: n_D^{20} 1.4848), MR_D 94.1. $C_{20}H_{32}O_2$; calc. 94.24; the substance had no absorption in the UV region at 220-375 nm. IR spectrum (ν, cm^{-1}): 3600-2400, 3020, 1710, 1640. PMR spectrum (δ, ppm): 0.85 (t, 3 H, CH_3), 1.30 (m, 6 H, CH_2—), 2.05 (m, 4 H, CH_2=C), 2.36 (m, 2 H, CH_2COO^-), 2.83 (t, 6 H, C=CCH_2C=C), 5.3 (t, 8 H, CH=CH, J = 4 Hz).

Prostaglandin E_2. Arachidonic acid (100 mg) in 10 ml of 50 mM Tris buffer (pH 8.0) was added to a mixture containing a microsomal preparation of prostaglandin synthetase (0.8 g of protein), 100 mg of epinephrine, and 80 mg of reduced glutathione in 80 ml of 50 mM Tris buffer (pH 8.0) that had previously been stirred at 35°C for 5 min. The reaction mixture was stirred at 35°C for 30 min with gassing by oxygen and was then cooled to 3-5°C, acidified with 4 M aqueous citric acid solution to pH 3.0, and extracted with ethyl acetate (3 × 100 ml). The extracts were washed with water and dried, and the solvent was distilled off. After adsorption chromatography on silica gel LS 100/160, 45.7 mg (39.5%) of a transparent oil was obtained.

PMR spectrum (δ, ppm): 0.83 (t, 3 H, CH_3), 1.27 (m, 8 H, CH_2—), 2.06 (m, 4 H, CH_2C=C), 2.30 (t, 2 H, CH_2COO^-), 4.10 (t, 2 H, OH), 5.36-5.55 (m, 4 H, HC=CH). Mass spectrum of the bistrimethylsilyl derivative of the methyl ester, m/z (%): 510 (1) (M^+), 439 (45) ($M^+ - C_5H_{11}$), 420 (24) ($M^+ - Me_3SiOH$), 366 (5) ($M^+ - 144$), 349 (77) ($M^+ - C_5H_{11} - Me_3SiOH$), 295 (100) ($M^+ - C_5H_{11} - 144$), 199 (25), 173 (5). After crystallization from ether containing hexane, the yield was 24.2 mg, mp 66-68°C (according to the literature: mp 65-67.5°C [7], mp 66-68°C [8]), $[\alpha]_D^{20}$ −67.3° (c 1; tetrahydrofuran). Mass spectrum with direct introduction at 100°C, m/z (%): 334 (28) ($M^+ - H_2O$), 316 (100) ($M^+ - 2H_2O$), 281 (6) ($M^+ - C_5H_{11}$), 263 (46) ($M^+ - C_5H_{11} - H_2O$), 245 (68) ($M^+ - C_5H_{11} - 2H_2O$), 217 (86) ($M^+ - C_5H_{11}CO - 2H_2O$).

SUMMARY

1. Methods have been developed for the isolation of arachidonic acid from the lipid wastes of endocrine production.

2. Prostaglandin E_2 has been obtained by enzymatic synthesis from the arachidonic acid isolated.

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