3. The presence of 2,3,4-tri-O-methylrhamnose, 3,4,di-O-methylrhamnose, 2,3,4,6-tetra-O-methylglucose, and 2,6-di-O-methylglucose and of a mixture of 2,3,6-tri-O-methylglucose and 2,3,6-tri-O-methylgalactose (1:10:14:5:7) in the products of the methylation of the polysaccharide from the bark of *A. nudiflora* after its reduction at the carboxy groups has been established.

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PREPARATION OF TRITIUM-LABELLED OLEIC ACID AND PROSTAGLANDINS E_2 AND $F_{1\alpha}$. A STUDY OF THE PROCESS OF INCLUDING TRITIUM IN MOLECULES OF UNSATURATED FATTY ACIDS AND PROSTAGLANDINS

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[^{3}H]Prostaglandin E_2 and [^{3}H]prostaglandin $F_{1\alpha}$ have been obtained with the aid of heterogeneous catalytic isotope exchange with gaseous tritium in solution. The distribution of the tritium in the labelled unsaturated fatty acids and prostaglandins has been studied.

We have previously shown the possibility of obtaining tritium-labelled saturated and unsaturated fatty acids by heterogeneous isotope exchange with gaseous tritium in the presence of transition metals [1, 2]. The process of introducing tritium into the molecules of natural compounds by this method is performed under mild conditions and does not require the preliminary protection of functional groups. By using this method we have obtained tritium-labelled eicosa-8,11,13-trienoic and arachidonic acids, which retained their biological activities, as was shown by the enzymatic conversion of these labelled acids into tritium-containing prostaglandins E_1 and E_2, respectively [2].

Isotope exchange between gaseous tritium and the hydrogen of unsaturated fatty acids is accompanied by the addition of hydrogen and of tritium at the double bonds. By suitable selection of the catalyst and of the reaction time it is possible to suppress the addition reaction to a considerable degree. Table 1 gives information characterizing the capacity of three batches of catalysts (A, B, and C) for effecting isotope exchange and also for adding hydrogen and tritium at the double bond of methyl oleate. For the subsequent investigations we used catalyst C, which gave the highest yield of labelled substance of the initial structure with a high specific activity. By isotope exchange on this catalyst we obtained tritium-labelled prostaglandin E_2 (PGE_2) and prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$).

On further investigation of the processes taking place during isotope exchange, we established that as well as addition reactions at least two other processes lowering the yield of desired product occurred: cis-trans isomerization and the migration of the double bond along the chain. In order to study these processes, the substances after isotope exchange with 80% tritium, were first purified by preparative TLC and were then separated on silica gel impregnated with silver nitrate. It has been shown previously [3] that under

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TABLE 1. cis-trans Isomerization and Tritiation of Methyl Oleate on Catalysts A, B, and C during isotope Exchange with 80% Tritium for 30 min

Reaction product	Specific activity, Ci/mmole (yield, %)		
	A	B	C
Methyl oleate	1,4 (26)	0,9 (45)	1,2 (40)
cis isomer	1,7 (31)	1,2 (42)	1,8 (35)
trans isomer	5,8 (10)	6,0 (3)	5,0 (6)
Methyl stearate			

TABLE 2. Migration of Double Bond during Itotope Exchange in Methyl Oleate with 80% Tritium for 1 h

Isotope exchange	Methyl oleate yield, %	
	cis isomer	trans isomer
Total	30	56
Without migration	29	29
With migration towards the carboxy group	<1	23
With migration towards the terminal methyl group	<1	4

these conditions the separation not only of the cis and trans isomers but also of the position isomers of cis-unsaturated acids takes place. In contrast to preceding investigations [2], in this case we analyzed all three zones of reaction products (Fig. 1). Zone I contained a substance of the initial structure, zone II products of the isomerization and migration of the double bond, and zone III hydrogenation products.

After isotope exchange with methyl oleate, labelled methyl oleate was detected in zone I, methyl trans-octadecenoate in zone II, and methyl stearate in zone III (Fig. 1). In its chromatographic mobility on silica gel impregnated with silver nitrate, the methyl ester on zone I proved to be identical with methyl elaidate. However, the periodate–permanganate oxidation of the substance from zone II followed by the gas-chromatographic analysis of the methyl esters of the monocarboxylic acids formed showed that this substance was inhomogeous and contained the methyl esters of three acids: elaidic (trans-octadec-9-enoic), trans-octadec-8-enoic, and trans-octadec-10-enoic (Table 2), which are not separated under the conditions of thin-layer chromatography.

In the case of the isotope exchange of PGE_2 with 80% tritium, zone I contained labelled PGE_2 and zone II contained labelled trans-PGE_2, which proved to be identical with the trans-PGE_2 described previously [4]. In its chromatographic behavior, the prostaglandin of zone III was identical with a sample of natural PGE_1. From this we concluded that the prostaglandin of zone III, like natural PGE_1, contained only a 13-trans double bond. This conclusion was also confirmed by the fact that under the same conditions $PGF_{1\alpha}$ scarcely formed either isomerization products or products of the addition of hydrogen and tritium to the double bond. The time of the reaction of PGE_2 with 80% tritium substantially affects the yield of labelled PGE_2. With an increase in the time of isotope exchange from 1 to 3 h the total yield of the three labelled prostaglandins fell threefold and the yield of labelled PGE_2 almost ninefold. Conversely, $PGF_{1\alpha}$ proved to be stable under the conditions of isotope exchange, forming labelled $PGF_{1\alpha}$ with a yield of 73% after 3 h (Table 3).

For the more complete characterization of the processes accompanying isotope exchange, we made a comparative investigation of the rates of the cis-trans isomerization methyl oleate $\rightleftarrows$ methyl elaidate, of the inclusion of the label in the cis and trans isomers, and of hydrogenation with 0.1% tritium. The ratio of the isomers in the reaction products was determined on the basis of the radioactivities of the cis and trans isomers formed, taking into account the ratio of the specific activities of the cis and trans isomers determined in a separate experiment for various intervals of time. The amount of methyl stearate in an aliquot was determined by the GLC method. It can be seen from the results obtained (Fig. 2) that the cis double bond isomerizes more readily than the trans double bond. In the case of methyl elaidate the cis isomer formed in the first 10-15 min does not accumulate in the reaction mixture but undergoes a further transformation, and by the end of the first hour of the reaction the amount of cis isomer has become so small that it can be neglected.

The processes of cis-trans isomerization and double-bond migration on metal catalysts can be described by two mechanisms — associative and dissociative — which have been confirmed by a large number of experimental results [5, 6]. According to the associative mechanisms, when an olefin is adsorbed on the surface of a catalyst the multiple bond is cleaved with the formation of carbon–metal and ordinary C–C bonds around which free rotation is possible.

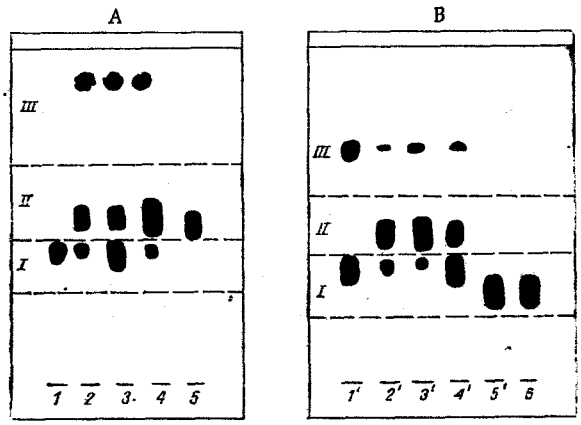


Fig. 1. Chromatography on silica gel impregnated with silver nitrate of the products of the isotope exchange reaction in methyl oleate (system A) (A) and in PGE_2 and $PGF_{1\alpha}$ (system C) (B): 1) methyl oleate; 2) reaction mixture; 3) mixture of methyl oleate and reaction products; 4) mixture of methyl elaidate and reaction products; 5) methyl elaidate; 1') PGE_2 and PGE_1; 2') reaction mixture after isotope exchange (1 h) in PGE_2; 3') reaction mixture after isotope exchange (3 h) in PGE_2; 4') mixture of PGE_2 and products of isotope exchange (1 h) in PGE_2; 5') reaction mixture after isotope exchange (3 h) in $PGF_{1\alpha}$; 6') $PGF_{1\alpha}$.

TABLE 3. Isotope Exchange with 80% Tritium in Methyl Oleate and in PGE_2 and $PGF_{1\alpha}$ on Catalyst C

Initial compound	Reaction time, h	Reaction product	Yield, %	Specific Activity, Ci/mmole
Methyl oleate	1	Methyl stearate	14	26.3
		cis Isomer	30	3.96
		trans Isomer	56	5.94
PGE_2	1	cis-PGE_2	26	1.74
		trans-PGE_2	43	2.09
		PGE_1	4	25.4
		cis-PGE_2	3	2.96
PGE_2	3	trans-PGE_2	14	3.16
		PGE_1	7	25.4
$PGF_{1\alpha}$	3	$PGF_{1\alpha}$	73	1.85

TABLE 4. Distribution of Tritium in the Fragments after Periodate-Permanganate Oxidation of Methyl Octadecenoates and the Free Acids

Compound	Amounts of tritium in the fragments, %			
	double bond	carboxyl part	alkyl part	
Methyl oleate	45		55	
Oleic acid	54	23		23
trans Isomers from methyl oleate	49		51	
trans Isomers from oleic acid	64	13		23

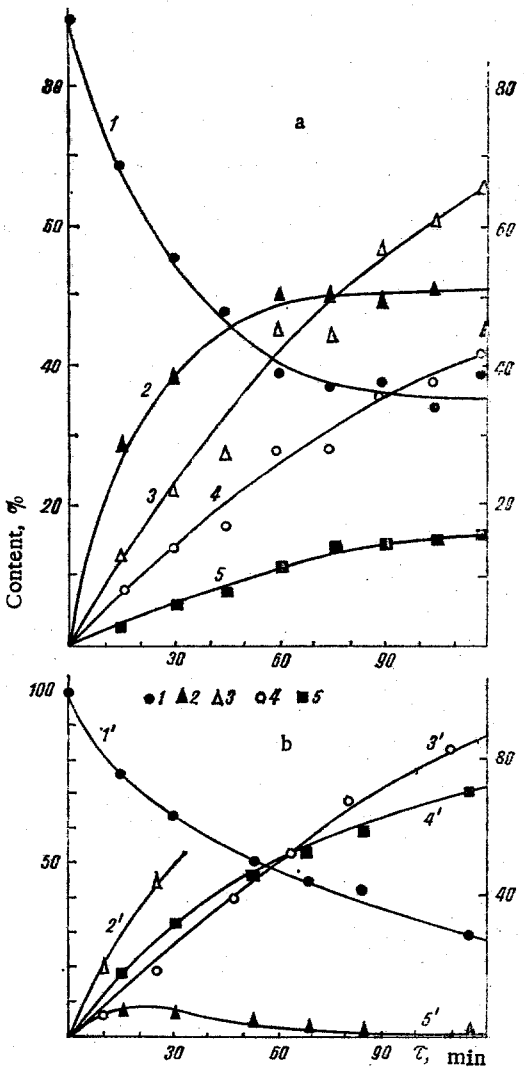
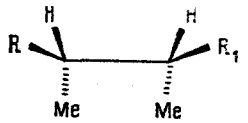


Fig. 2. Processes taking place during isotope exchange between 0.1% tritium and methyl oleate (a) and between 0.1% tritium and methyl elaidate (b): 1) change in the amount of cis-$C_{18:1}$; 2) change in the amount of trans-$C_{18:1}$; 3) inclusion of the tritium label in trans-$C_{18:1}$; 4) inclusion of the tritium label in cis-$C_{18:1}$; 5) change in the amount of $C_{18:0}$; 1') change in the amount of trans-$C_{18:1}$; 2') inclusion of the tritium label in trans-$C_{18:1}$; 3') inclusion of the tritium label in trans-$C_{18:1}$; 4') change in the amount of $C_{18:0}$; 5') change in the amount of cis-$C_{18:1}$.

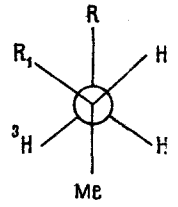
The dissociative mechanism predicates the detachment of hydrogen from the olefin molecule with the formation of a carbon—metal bond. If the hydrogen is split out from the α-position to the double bond, a radical of the π-allyl type is formed, and in this case free rotation of the alkyl part of the molecule is impossible [5]. Thus, by the associative mechanism it is mainly cis-trans isomerization which takes place, while the dissociative mechanism describes mainly the migration of the double bond, and also the inclusion of the label in the saturated parts of the molecule.



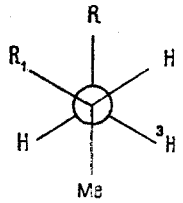
a $R_1=C_7H_{14}COOCH_3, R=C_8H_{17}$

b $R_1=C_8H_{17}, R=C_7H_{14}COOCH_3$

I a, b



II a, b



III a, b

IV a, b

a) $R_2=C_6H_{12}COOCH_3, R_3=C_7H_{15}$

b) $R_2=C_7H_{15}, R_3=C_6H_{12}COOCH_3$

Let us consider the cis-trans isomerization of the double bond in methyl oleate from the point of view of the associative mechanism. After the adsorption of the methyl oleate on the surface of the catalyst and the formation of two carbon—metal bonds with the simultaneous opening of the double bond (I), an attack by hydrogen or tritium of one of the two carbon atoms bound to the catalyst takes place. In this process, the formation of two semihydrogenated forms is possible which adopt hindered conformations with the rotation of the radical R around the $C_{(9)}$—$C_{(10)}$ bond (conformations II and III). Then the hydrogen or tritium is split off from the semihydrogenated form with the simultaneous cleavage of the carbon—metal bond. This leads to the reformation of the double bond, which may now have either the cis or the trans configuration. In the case of methyl oleate, the ratio of cis and trans isomers of the Δ9 position is 1:1, but in an analysis of the distribution of the label in the cis and trans isomers it was found that the double bond of the trans isomer contained more tritium than the double bond of the cis isomer (Table 4). This witnesses dissimilar probabilities of conformations II and III with a predominance of II (in the case of absorption of the π-allyl type the migration products do not contain tritium at the double bond).

The presence of migration products only with the trans configuration of the double bond (Table 4) shows that this process takes place not by the associative but by the dissociative mechanism. By the dissociative mechanism, after the detachment of a proton from the α-position to the double bond in methyl oleate, an intermediate radical of the π-allyl type (IV) is formed which, after rearrangement and the addition of hydrogen or tritium, should give only methyl trans-octadecenoate with the label included only in the α-position to the double bond. Because of the asymmetry of the molecule of a methyl ester of a fatty acid, migration of the double bond takes place unequally in the two directions (Table 2). In the case of methyl oleate, migration takes place predominantly towards the carboxy end of the molecule, which, apparently lies closer to the surface of the catalyst because of the additional adsorption of the carboxy group.

It can be seen from Table 2 that, as was to be expected, in the reaction of the double bond on the catalyst both mechanisms are realized simultaneously. Whether the associative

TABLE 5. Distribution of Tritium in the Fragments after the Periodate–Permanganate Oxidation of Methyl Arachidonate, Arachidonic Acid, and PGE_2 and $PGF_{1\alpha}$

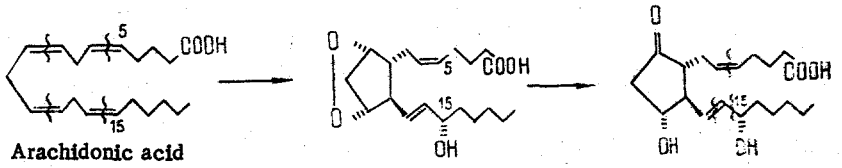
Compound	Amount of tritium in porportions of the specific activity, Ci/mmole (%) in the fragments				
	double bond	carboxylic moiety	alkyl moiety	fragment with the cyclopentane	malonic acid
Methyl arachidonate	0.35 (14)	—	1.18 (47)	—	0.98 (39)
Arachidonic acid	0.36 (27)	0.16 (12)	0.38 (28)	—	0.44 (33)
PGE_2 from biosynthesis	0.05 (7)	0.20 (26)	0.39 (51)	0.12 (16)	—
PGE_2 1 h*	0.52 (30)	0.38 (22)	0.52 (30)	0.31 (18)	—
PGE_2 3 h*	1.07 (36)	0.50 (17)	1.07 (36)	0.33 (11)	—
trans isomer from PGE_2, 1 h*	1.04 (54)	0.22 (12)	0.43 (23)	0.20 (11)	—
trans isomer from PGE_2, 3 h*	1.40 (50)	0.31 (11)	0.81 (29)	0.28 (10)	—
$PGF_{1\alpha}$	0.26 (14)	—	0.98 (53)	0.61 (33)	—

*Time of isotope exchange.

or the dissociative mechanism predominates apparently depends on the structure of the reacting molecule.

To evaluate the distribution of the tritium in the molecules of labelled compounds we performed the periodate–permanganate oxidation of arachidonic and octadecenoic acids and their methyl esters and also of samples of PGE_2 obtained by biosynthesis from labelled arachidonic acid and by isotope exchange and of $PGF_{1\alpha}$. The amount of tritium at the double bond was calculated from the decrease in radioactivity of the mixture of reaction products after oxidation in comparison with the radioactivity of the initial substance. The radioactivities of the fragments formed by the oxidation of a labelled compound were determined after their separation by thin-layer chromatography (Tables 4 and 5). It has been reported previously [2] that when methyl arachidonate is saponified its specific activity falls. Analysis of the distribution of tritium in methyl arachidonate and arachidonic acid showed that on saponification practically none of the tritium present at the double bond is lost, while tritium attached to the carbon atoms occupying the α-positions in relation to the double bond and to the carboxy group undergoes active exchange with the protons of the medium under these conditions. Similar results have been obtained for the octadecenoic acids and their methyl esters (Tables 4 and 5).

A comparison was made of the distributions of the label in [^{3}H]arachidonic acid, the [^{3}H]PGE_2 obtained by biosynthesis from this acid, and the [^{3}H]PGE_2 obtained as the result of isotope exchange. It is known that in the biosynthesis of the prostaglandins there is a rearrangement of the part of the arachidonic acid molecule from $C_{(8)}$ to $C_{(15)}$ [7] (see scheme; the wavy lines in the scheme show the positions of cleavage of the bonds on oxidation with the permanganate–periodate mixture). It has been reported



Arachidonic acid PGE 2

previously that [^{3}H]PGE_2 obtained by biosynthesis had a smaller specific activity than the initial [^{3}H]arachidonic acid (0.77 and 1.34 Ci/mmole, respectively) [2]. Analysis of the distribution of the label in these substances (Table 5) showed that the decrease in specific activity is connected with the loss of tritium from those carbon atoms that participate in the reaction forming the prostaglandin structure, while the label in the other parts of the arachidonic acid molecule is retained almost completely. Thus, for example, the contributions to the total specific activity of the alkyl and carboxylic moieties of the molecules

of arachidonic acid and PGE_2 were almost the same (Table 5 and the Scheme). The PGE_2 labelled by isotope exchange and the [^{3}H]PGE_2 obtained by biosynthesis from [^{3}H]arachidonic acid differed greatly from one another both in specific activity (which was almost four times higher for the former) and in the distribution of the tritium (see Table 5). Since with a lengthening of the time of isotope exchange the inclusion of tritium increases mainly on the double bond and in the alkyl moiety of the PG molecule (Table 5), it is possible to obtain PGE_2 differing still more greatly from the biosynthetic PGE_2 with respect to the distribution of the label, and this can be used in the study of the mechanism of the physiological action of the prostaglandins and of their metabolism.

EXPERIMENTAL

For the investigation we used methyl esters of commercial oleic and elaidic acids with a purity of not less than 98% (according to GLC (isothermal, 170°C). The $PGF_{1\alpha}$ and PGE_1 were kindly supplied by Dr. J. Pike (Upjohn, USA). The PGE_2 was obtained by biosynthesis from arachidonic acid. The solvents were purified by standard methods.

GLC was performed on a Varian 2100 chromatograph fitted with a 2 × 2000 mm column containing 3% of SE-30/Chromosorb W-HP (80-100 mesh), with a rate of flow of helium of 30 ml/min. The UV spectra were measured on a Specord UV-VIS spectrometer. Radioactivities were measured with a scintillation counter having a recording efficiency for tritium of 12% in a dioxane scintillator [8] containing 2% of acetic acid.

Preparative TLC was performed on 13 × 18 cm plates with a fixed layer of Kieselgel V silica gel containing 10% of gypsum, or on the same plates impregnated with a 12% solution of silver nitrate. Analytical TLC was performed on Silufol plates or on the same plates impregnated immediately before use by a single spraying with a 12% solution of silver nitrate in 95% aqueous methanol followed by drying with hot air for 5 min.

For TLC we used the following systems: 1) hexane—ether (9:1); 2) toluene—dioxane—acetic acid (20:20:1); 3) benzene—ethyl acetate—dioxane—acetic acid (10:10:10:1); and 4) chloroform—methanol—4 N ammonia (65:25:4). The spots were revealed with a 10% solution of tungstophosphoric acid in methanol, and then by treatment with a methanolic solution of sulfuric acid (dilution 1:100) followed by heating. Solutions were evaporated in vacuum in a rotary evaporator at a bath temperature not exceeding 30°C. The palladium catalysts A, B, and C were obtained by a standard procedure [9].

The isotope exchange reaction was performed in tubes on apparatuses giving 0.1 and 80% tritium [1] at a molar ratio of palladium to substance of 1:1 in dioxane (the tubes were filled with the tritium-hydrogen mixture to a pressure of 250 mm Hg). The methyl esters of the octadecenoic acids were extracted from the catalysts with methanol, the methanol was eliminated, and the residue was evaporated several times with methanol to eliminate labile tritium.

The $PGF_{1\alpha}$ and PGE_2 were extracted from the catalyst with chloroform—methanol—water (5:5:1), the solvent was driven off, and the residue was evaporated several times with the same mixture to eliminate labile tritium. The $PGF_{1\alpha}$ and PGE_2 were freed from degradation products on preparative plates in system 2. The products of isotope exchange were separated by preparative TLC on plates with silver-nitrate-impregnated silica gel (by a method described previously [2]) in system 1 for methyl oleate and methyl elaidate and system 3 for $PGF_{1\alpha}$ and PGE_2 (Figs. 1 and 2). The homogeneity of the products obtained was checked by TLC on silver-nitrate-impregnated Silufol plates. The reaction times and the yields and specific activities of the products are given in Tables 1 and 3. The tritium-labelled methyl octadecenoates were saponified with a 1:1:1 mixture of 6% aqueous potassium hydroxide, methanol, and dioxane, as described previously.

In this way we obtained: [^{3}H]oleic acid with a yield of 95% (radio activity yield 84%) and a specific activity of 3.32 Ci/mmole, and [^{3}H]-trans-isomers from oleic acid with a yield of 95% (75%) and a specific activity of 4.46 Ci/mmole.

Periodate—Permanganate Oxidation. To study the distribution of tritium in the labelled compounds, 240 µl of water, 240 µl of oxidizing mixture (containing 97 mg of sodium periodate and 7.9 mg of potassium permanganate per 5 ml of water), and 40 µl of 0.02 M potassium carbonate solution were added to a solution of 100 µg of the substance in 160 µl of tert-butanol. The mixture was shaken vigorously at room temperature for 2 h, and then an excess of ethylene glycol was added and the reaction mixture shaken again until the color

had disappeared, after which it was diluted with methanol to 1 ml. Aliquots (1-4 μl) of this mixture were taken and were placed in five small bottles. To the first bottle was added scintillator, and the initial radioactivity was measured. To each of the remaining bottles was added 1 ml of methanol and the contents were then evaporated to dryness. After this, to each of the bottles except the second 1 ml of methanol was added and this was again evaporated. To the second bottle was added 1 ml of benzene, and this was evaporated off and scintillator was added. Then the cycle was repeated again with the remaining bottles until they had all been treated. After the measurement of the radioactivity, it was established that the complete elimination of tritium-containing water required an aliquot to be evaporated twice with methanol and then once with benzene. A comparison of radioactivities before and after the elimination of the tritium-containing water gave the amount of label present at double bonds (in the case of the prostaglandins it also included the tritium attached to the 15th carbon atom).

To the remainder of the reaction mixture after evaporation 1 ml of a mixture of a saturated solution of sodium chloride and water (1:1) was added in a current of argon and the reaction products were extracted with ethyl acetate (3 × 1 ml). The extract was washed with saturated sodium chloride solution and was evaporated in a current of argon. The residue was dissolved in 500 μl of ethyl acetate, and aliquots (1-4 μl) were separated by TLC on Silufol plates (Czechoslovakia) in system 4. After the extraction of the substances from the silica gel with 5 ml of scintillator over 2 h, we determined the radioactivities of the zones corresponding to monocarboxylic acids (alkyl moiety) (R_f 0.6-0.9), to dicarboxylic acids (carboxylic moiety) (R_f 0.2-0.3), to malonic acid (for arachidic acid and methyl arachidonate) (R_f < 0.15), and to the fragment containing the cyclopentane ring (for PGE_2, R_f < 0.1, and for $PGF_{1\alpha}$ R_f 0.4-0.55).

To study the migration of the double bonds in the methyl octadecenoates, to the reaction mixture after the periodate–permanganate oxidation of 1-2 mg of substance and working up as described above was added 1 ml of a mixture of a saturated solution of sodium chloride and 0.1 N hydrochloric acid (1:1), and extraction was carried out with ethyl acetate (3 × 1 ml). The extract was dried with sodium sulfate, filtered, and evaporated at −30°C in a current of argon. The residue was methylated with a small excess of diazomethane, and the solvent was eliminated at −50°C. The methyl esters obtained, in the form of a solution in chloroform, were analyzed by GLC under isothermal conditions (70°C) and with programming of the temperature from 50 to 200°C.

SUMMARY

[^{3}H]Prostaglandin E_2 and [^{3}H]prostaglandin $F_{1\alpha}$ have been obtained by heterogeneous catalytic isotope exchange with gaseous tritium in solution. Experiments with methyl oleate, methyl elaidate, and prostaglandin E_2 have shown that, as well as isotope exchange on Lindlar catalyst, the addition of hydrogen and tritium to the double bonds and isomerization and migration of the double bonds take place. The distribution of the tritium in the labelled unsaturated fatty acids and prostaglandins has been studied. It has been established that the [^{3}H]prostaglandin E_2 obtained by biosynthesis from labelled arachidonic acid differs in relation to the distribution of the label from the [^{3}H]prostaglandin E_2 obtained by the method of isotope exchange.

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