J. Lipid Research
October, 1959

Positional distribution of saturated and unsaturated fatty acids on egg lecithin*

N. H. Tattrie

Division of Applied Biology, National Research Council, Ottawa 2, Canada

[Received for publication May 21, 1959]

SUMMARY

A method is described to determine the position of fatty acids on lecithin. Purified egg lecithin is enzymatically hydrolyzed to the corresponding mixture of α,β-diglycerides with lecithinase D. Myristic acid is incorporated into the α' position of the diglycerides and the resulting triglycerides are hydrolyzed with pancreatic lipase, which specifically cleaves fatty acids from the α and α' positions. Palmitic, myristic, and stearic acids were freed by the pancreatic lipase, proving that the saturated acids are on the α' position, while the unsaturated fatty acids occupy the β position of egg lecithin. Since snake venom lecithinase A removes the unsaturated acids from lecithin, the site of hydrolysis must be at the β position of lecithin.

It has been known for several years that snake venom lecithinase A liberates unsaturated fatty acids from lecithins containing both saturated and unsaturated fatty acids (1). In recent years this observation has been confirmed in more detail by several investigators and the resulting lysolecithin has been shown to contain mostly saturated fatty acids (2 to 5). These observations cannot be explained on the basis that lecithinase A is specific for the hydrolysis of only unsaturated fatty acid ester linkages, since both di-saturated and di-unsaturated lecithins have been shown to be hydrolyzed equally well to yield one mole of fatty acid and the corresponding lysolecithin (6). It was therefore concluded (7) that the action of the enzyme was directed toward one particular ester group, α' or β, in the lecithin molecule.

Hanahan (8) attempted to find the site of attack of the enzyme by determining the position (α' or β) of the free hydroxyl group in lysolecithin. After oxidation of the lysolecithin with permanganate, followed by acid hydrolysis, he isolated phosphoglyceric acid in 65 to 85 per cent yield as the only detectable phosphate ester and concluded that the free hydroxyl group was in the α' position; therefore the lecithinase A specifically hydrolyzed the ester linkage in the α' position.

Long and Penny (9) reached the same conclusion when they found that lysolecithin was oxidized by acid dichromate with an uptake of four equivalents, as expected for a compound containing a primary hydroxyl group. Later Davidson *et al.* (10) isolated an acidic product of the permanganate oxidation of lysolecithin and showed it to be lysolecithinic acid. Gray (11) has recently confirmed this finding. Neither of the latter groups, however, reported any yields for lysolecithinic acid, nor did they look for the presence of other phosphate esters in the products of oxidation.

Marinetti *et al.* (12) have recently repeated Hanahan's oxidation procedure but obtained evidence for the presence of a keto-lysolecithin as well as lysolecithinic acid. After acid hydrolysis of the oxidation mixture, four water-soluble phosphate esters were detected: glycerophosphate, phosphoglyceric acid, phosphorylcholine, and orthophosphate. They concluded that lysolecithin was a mixture of α and β isomers, and that the enzyme hydrolyzes ester linkages at both the α' and β positions. Marinetti *et al.* (13) also found that snake venom lecithinase A would hydrolyze ester linkages from the β position of beef heart plasmalogens. They interpreted this latter finding as further proof for the lack of specificity of lecithinase A.

McArthur and Bennett also repeated the oxidation of snake venom-produced lysolecithin with permanganate and found that 74.5 hours at 37.5°C were necessary for the uptake of 2 atoms of oxygen per mole of lysolecithin. Methylglyoxal was present after acid hydrolysis but they were unable to detect any phos-

* Issued as N.R.C. No. 5379.

phoglyceric acid. They concluded that the lysolecithin must have been predominately the α' acyl lysolecithin.[1]

In view of the conflicting results obtained by permanganate oxidation procedures, it appeared desirable to try an entirely different and independent approach to the problem of the specificity of lecithinase A. Basically, the question of the specificity of lecithinase A, at this stage, amounts to the positional arrangement of the fatty acids in the lecithin used as a substrate. In recent years the determination of the positional arrangement (α or β) of fatty acids in triglycerides has been made possible by the discovery that pancreatic lipase initially catalyzes the hydrolysis of only primary ester linkages (14, 15, 16). Therefore by converting purified egg lecithin to α,β-diglycerides—by the action of lecithinase D (17)—introducing a known fatty acid at the primary hydroxyl group of these diglycerides, subjecting the resulting triglycerides to the action of pancreatic lipase, and analyzing the free acids liberated, it should be possible to determine which fatty acids were originally present on the α' and β positions of lecithins (Fig. 1). Comparison with the fatty acids liberated by lecithinase A should then establish which fatty acid-ester linkages are cleaved by this enzyme (Fig. 2).

The present paper provides new evidence on the position of saturated and unsaturated fatty acid esters in purified egg lecithin.

EXPERIMENTAL

Analytical Methods. Phosphorus was determined by King's method (18), total nitrogen by a micro-Kjeldahl procedure (19), and total choline by Glick's method (20).

Fatty acid analysis was carried out as follows: The lipid sample (150 mg.) was saponified with 10 ml. of aqueous N NaOH by reflux on a steam bath for 3 hours. After cooling, the mixture was acidified with 50 ml. of 5 N HCl. The fatty acids were extracted with ethyl ether and the ethereal solution washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* at 35°C in a stream of N_2. The residue was dissolved in hot methanol: water (9:1; v/v) and titrated with 0.02N NaOH in 90 per cent aqueous methanol to the o-cresol red end point. After acidification, the free fatty acids were extracted with petroleum ether (B.P. 30-60°C), concentrated in a stream of N_2, dried over P_2O_5 *in vacuo*, and weighed. The acids were then dissolved in ethyl ether, converted to the methyl esters with diazomethane, and analyzed by gas-liquid partition chromatography.

Gas-liquid partition chromatography was carried out on a Podbielniak "chromacon" (Series 9475-3V), with a thermal conductivity detector, which had been

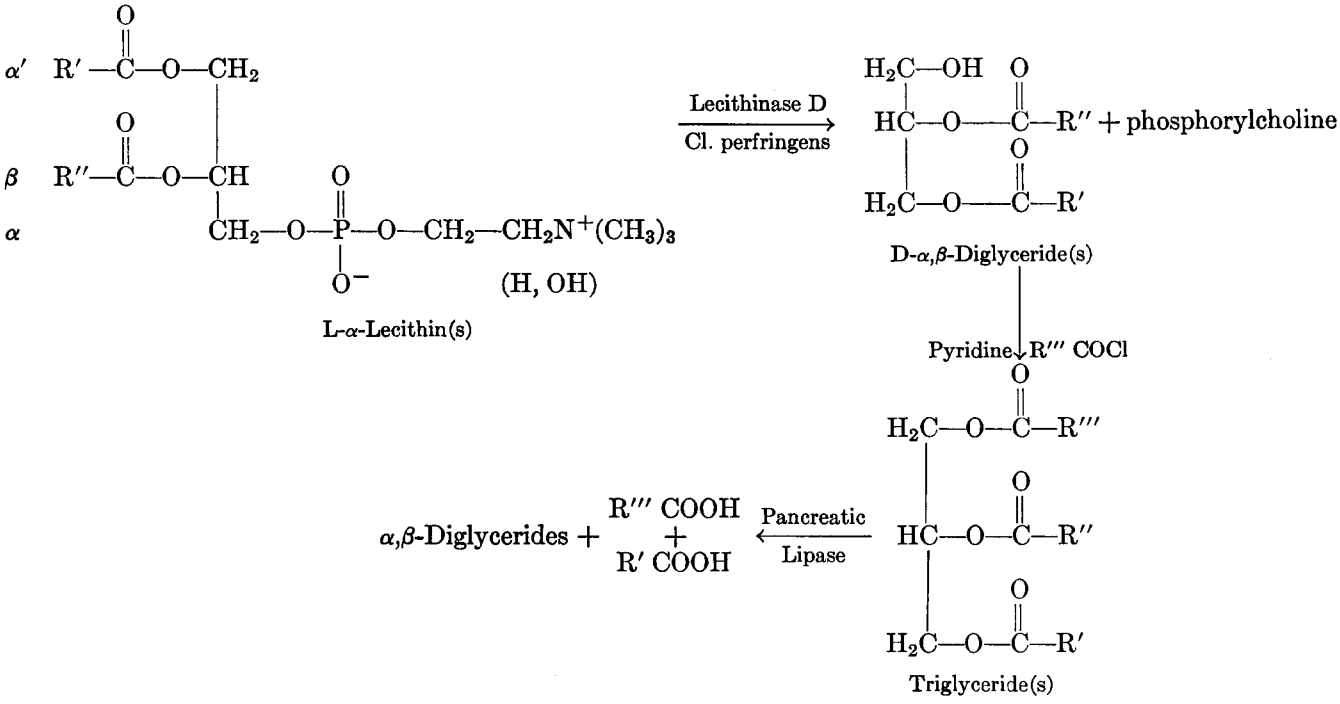

FIG. 1. Reaction scheme for the proof of positional distribution of fatty acids on lecithin.

[1] Communication from Dr. C. S. McArthur and Mr. J. R. Bennett, University of Saskatchewan, Saskatoon, Sask.

modified to take glass columns and to improve the pressure regulation, temperature control, and collection and injection systems. The glass chromatographic columns (6 mm. internal diameter by 8 feet length) were packed with Celite 545 impregnated with a polyester of 1,4-butanediol succinate (7/3; w/w) by the method of Craig.[2] Helium was used as a carrier gas.

No attempts were made to recover all the fatty acid esters from the chromatogram. The amount of each fatty acid ester was determined by measuring the peak area, a factor converting peak area to moles percentage of each of the acids having been previously determined with a known mixture.

Preparation of Egg Lecithin. Egg lecithin was prepared by the method of Rhodes and Lea (21) using alumina and silicic acid columns. Yield of acetone-precipitated lecithin was 9.85 g. per dozen fresh eggs. The final product was found to be chromatographically homogeneous on silicic acid-impregnated paper when stained with phosphomolybdic acid (22). The fatty acid composition of "purified lecithin" is given in Table 1, from which the mean molecular weight was calculated to be 273. On analysis the lecithin gave the following values for: N, 1.82; P, 3.94; C, 64.93; H, 10.74; ester choline, 15.27; mean molecular weight of fatty acids, 276; total fatty acids, 70.3 per cent; N to P molar ratio, 0.98; fatty acid to P molar ratio, 1.99; choline to P molar ratio, 0.99; calculated for $C_{42.5}H_{85}O_9NP$ (m.w. 785): N, 1.78; P, 3.95; C, 65.04; H, 10.87; ester choline, 15.44; mean molecular weight of fatty acids, 273; total fatty acids, 69.5 per cent.

Preparation of Lysolecithin. Egg lecithin (150 mg.), containing 5.95 mg. of P., was dissolved in 100 ml. of diethyl ether and treated with 1 ml. of 0.1 per cent rattlesnake venom (*Crotalus adamanteus*) in 0.005 M $CaCl_2$ solution (23). The solution was allowed to stand overnight at room temperature, the precipitate was removed by centrifugation and washed once with diethyl ether. No phosphorus was present in the ether phase, indicating that the reaction was complete. The free fatty acids in the ether phase and those remaining in the lysolecithin were analyzed by gas-liquid partition chromatography (Table 1 and Fig. 4). The lysolecithin was chromatographically homogeneous on silicic acid-impregnated paper when stained with phosphomolybdic acid (22).

Preparation of d-α, β-diglyceride. Purified lecithin was converted to the corresponding α,β-diglycerides by the method of Hanahan and Vercamer (17) using *Cl. perfringens*.[3] From 1 g. of egg lecithin, 748 mg. of

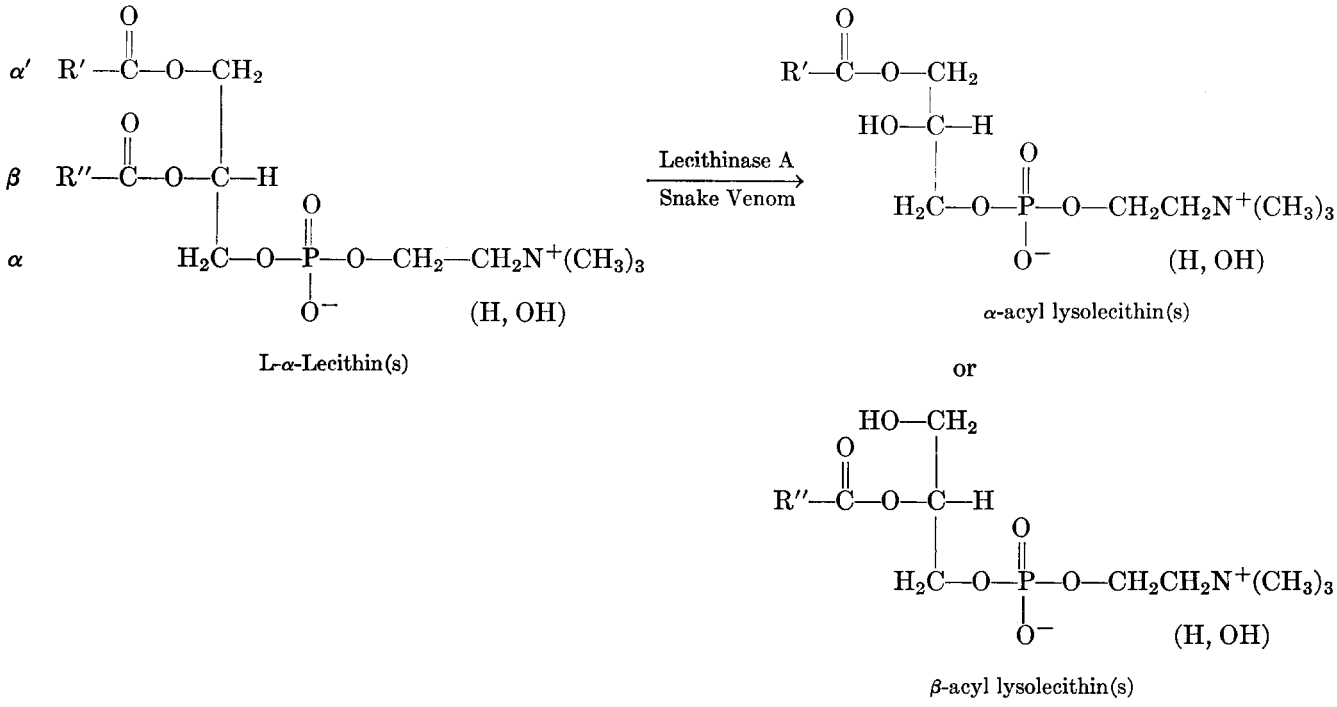

FIG. 2. Reaction scheme for the action of snake venom lecithinase A on lecithin.

[2] Communication from Dr. B. M. Craig, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.

[3] Supplied by Dr. D. J. Kushner, Forest Insect Laboratory, Sault Sainte Marie, Ont.

TABLE 1. FATTY ACID COMPOSITION OF VARIOUS FRACTIONS AND COMPOUNDS

Compound	Moles Per Cent Acid				
	Myristic	Palmitic	Stearic	Oleic	Linoleic
1. Egg lecithin	—	35.7	14.9	37.0	12.4
2. Lysolecithin (formed from egg lecithin by lecithinase A)	—	69.7	30.3	—	—
3. Fatty acids (freed from egg lecithin by lecithinase A)	—	—	—	74.8	25.2
4. α,β-diglyceride (formed from egg lecithin by lecithinase D)	—	35.1	14.6	36.8	13.5
5. Triglyceride (formed from α,β-diglyceride by acylation with myristoyl chloride)	32.1	23.9	9.9	25.0	9.1
6. Fatty acids (freed from above triglyceride by pancreatic lipase in 15 minutes)	48.1	36.0	15.9	—	—
6a. Fatty acids (freed from above triglyceride by pancreatic lipase in 25 minutes)	40.1	30.2	13.3	12.1	4.3
7. Fatty acids (freed from 2-oleoylpalmitoyl stearin by pancreatic lipase in 15 minutes)	—	48.3	51.7	—	—
7a. Fatty acids (freed from 2-oleoylpalmitoyl stearin by pancreatic lipase in 25 minutes)	—	45.2	46.1	8.7	—

a slightly yellow oil (free of phosphorus) was obtained (theory, 765 mg.). The infrared spectrum of the oil was similar to that of a synthetic d-α,β-dipalmitin (24). Total fatty acids were determined by gas-liquid partition chromatography (Table 1).

Preparation of Triglyceride from α,β-diglyceride. The d-α,β-diglycerides (500 mg.) formed from egg lecithins were acylated with myristoyl chloride in the presence of pyridine, as described in a previous publication (16). After isolation of the triglycerides from the acylation mixture, the dried yellow oil weighed 633 mg. (theory, 676 mg.). The oil was dissolved in 3 ml. of benzene and applied to a column of 15 g. of silicic acid (Mallinckrodt, 100 mesh, prepared by the method of Hirsch and Ahrens (25) to remove fine particles). Elution was continued with benzene and 10 ml. fractions were collected until no more lipid was eluted. After removal of the solvent *in vacuo,* in the presence of N_2, 604 mg. of an almost colorless liquid was recovered. The infrared spectrum was similar to that of a synthetic oleoyldipalmitin. No attempts were made to crystallize the triglyceride mixture. Total fatty acids were analyzed by gas-liquid partition chromatography (Table 1).

Hydrolysis of Triglycerides with Pancreatic Lipase. Synthetic 2-oleoylpalmitoylstearin [4] was used as a substrate in order to test the validity of using pancreatic lipase to cleave specifically fatty acids from the primary positions of triglycerides. Two hundred mg. of the synthetic triglyceride and 200 mg. of the triglycerides formed from the α,β-diglycerides were each hydrolyzed for 15 and 25 minutes consecutively, the free fatty acids were isolated as previously described (16) and estimated as methyl esters by gas-liquid partition chromatography. The results are summarized in Table 1 and Figure 3.

RESULTS AND DISCUSSION

Purified egg lecithin contains four fatty acids, palmitic, stearic, oleic, and linoleic in the molar ratios of 1.0, 0.41, 1.04, and 0.35 (Table 1). In addition to these acids, traces of myristic, palmitoleic, a C_{20}, and a C_{22} acid have been observed, but they were disregarded in the present paper.

As has been previously observed by a number of

[4] Supplied by Dr. W. Landmann, U.S. Department of Agriculture, New Orleans 19, La.

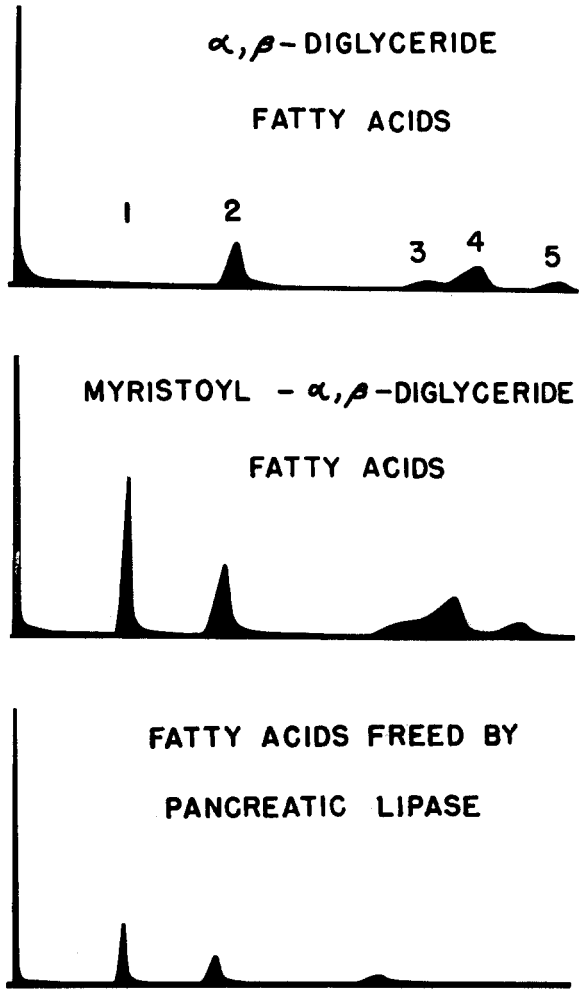

FIG. 3. Separation of methyl esters by gas-liquid partition chromatography, showing the action of pancreatic lipase on myristoyl-α,β-diglyceride. Peak identification: *1*, myristate; *2*, palmitate; *3*, stearate; *4*, oleate; *5*, linoleate.

investigators (2 to 5), snake venom lecithinase A removed only the unsaturated fatty acids, oleic and linoleic, from egg lecithin and left the saturated fatty acids, palmitic and stearic, in the lysolecithin (Table 1 and Fig. 4). There did not appear to be any unsaturated acids remaining in the lysolecithin, although previous workers have observed 0.15 double bonds per mole (4). However, methyl stearate and methyl oleate are not completely separable on the chromatogram and a trace of the latter may have been obscured by the large peak for the C_{18} saturated acid.

When synthetic 2-oleoylpalmitoylstearin was enzymatically hydrolyzed with pancreatic lipase for 15 minutes only palmitic and stearic acids (1:1) were released (Table 1), but if the reaction time was allowed to proceed for 25 minutes, approximately 10 per cent oleic acid appeared. The release of fatty acids originally in the β position of the triglyceride probably indicates hydrolysis of the diglyceride. It is therefore important to standardize the reaction time when using this enzyme to cleave specifically fatty acids from the primary positions of triglycerides.

When the triglycerides formed from lecithin were hydrolyzed by the pancreatic lipase for 15 minutes, only myristic, palmitic, and stearic acids were released (Table 1 and Fig. 3). Furthermore, the mole ratio of myristic acid to palmitic plus stearic acid was very close to 1. This is unequivocal evidence that only saturated fatty acids are present in the α′ position and that the unsaturated fatty acids are in the β position of egg lecithin. Since snake venom lecithinase A removes only unsaturated fatty acids, and, as shown above, these occur on the β position, the lysolecithin must be the α′ acyl ester. This finding is contrary to the conclusions of Hanahan (8) and of Long and Penny (9), but the possibilities of acyl migration, occurring during enzymatic hydrolysis of lecithin, or the oxidation of lysolecithin, or both, were not elim-

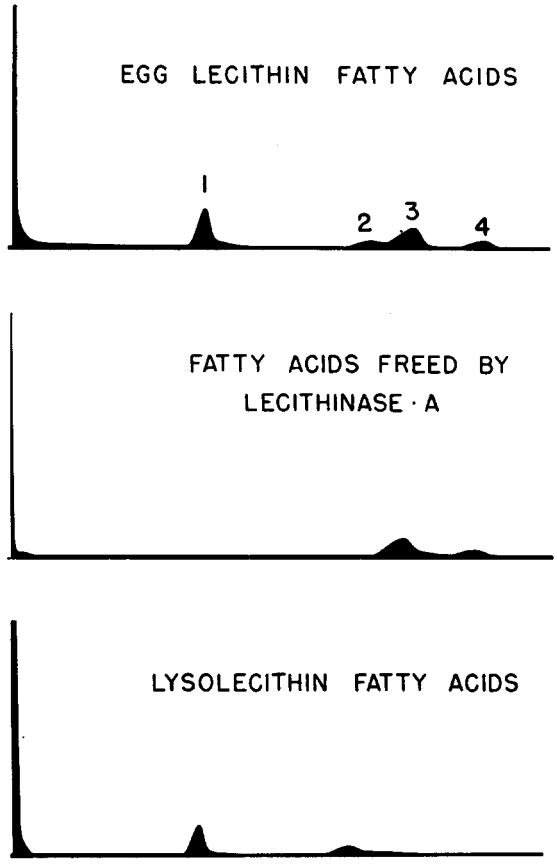

FIG. 4. Separation of methyl esters by gas-liquid partition chromatography, showing the action of lecithinase A on lecithin. Peak identification: *1*, palmitate; *2*, stearate; *3*, oleate; *4*, linoleate.

inated in their experiments. The present method, on the other hand, is based on reactions in which acyl migration is highly unlikely. The action of lecithinase D on lecithin has been shown to produce a d-α,β-diglyceride without racemization (17, 26), and acylation in the presence of pyridine does not cause acyl migration (27).

Mattson and Lutton (28), as well as Savary and Desnuelle (29), have recently found that there is some degree of specificity in the arrangement of fatty acids in glycerides. They found that most glycerides have unsaturated fatty acids in the β position. The finding that egg lecithin also has unsaturated fatty acids in the β position suggests that there may be a direct relation between the biosynthesis of triglycerides and phospholipids.

The author is deeply indebted to Mr. A. G. McInnes and Dr. M. Kates for their valuable advice. Sincere thanks are also expressed to Mr. F. Cooper for all the gas-liquid partition chromatographic analyses. The author expresses his gratitude to Mr. A. Castagne for C, H, and N analyses.

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