[JOINT CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1] AND THE RADIOCARBON LABORATORY, UNIVERSITY OF ILLINOIS]

Labeling Fatty Acids by Exposure to Tritium Gas. II. Methyl Oleate and Linoleate[2]

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Received January 15, 1960

Unsaturated fatty acid esters react at room temperature on exposure to gaseous tritium by addition of tritium to a double bond and with little or no substitution of tritium for hydrogen. Evidence for addition to olefinic bonds, rather than substitution for hydrogen, has been obtained from gas-liquid and liquid-partition chromatography of both the tritiated fatty acids and the tritiated products after mild oxidative cleavage. Tritiated fatty acid esters appear on chromatograms at positions of the next less-saturated member of the isologous series. The position of addition of the tritium is deduced from the radioactivity of the monobasic and dibasic acids produced by oxidation.

Tritium labeling of methyl esters of saturated fatty acids by the Wilzbach gas-exposure technique[5] is described in the first paper[6] of this series. The expected substitution of hydrogen by tritium gas was found to take place giving the desired labeled fatty acids with high specific activity and with only small amounts of radiation-induced impurities. Results are presented herein on gas exposure and analysis of two members of the C_{18} unsaturated fatty acid ester series—methyl oleate and linoleate—in which addition to the double bond, not substitution, is found to occur. This discovery not only holds significance to those interested in the labeling of unsaturated fatty acids but shows the need for caution when labeling olefinic compounds by the gas-exposure technique and for establishing the type of labeling which has taken place.[7]

RESULTS

Data on the irradiation and chemical purification of methyl oleate and linoleate are given in Table I. A tritium source containing approximately 1 curie was employed for the irradiation of gram samples of the methyl esters. Activities incorporated in an 18-day period were 8–14 mc. Of this total radioactivity, 76% to 82% was recovered in the purified fatty acid fraction. In agreement with earlier results[6] for saturated fatty esters, little activity remained in the acid aqueous layer after ether extraction. Also activity in the unsaponifiable fraction was found to be due partly to labile tritium exchanged by the ethanol present in the ether layer. The effectiveness of chemical purification procedures in removing labile tritium, and evidence for the structure of the tritiated methyl esters will be given in the following sections.

TABLE I

TRITIUM INCORPORATION AND DISTRIBUTION IN METHYL OLEATE AND METHYL LINOLEATE BY THE GAS EXPOSURE TECHNIQUE

Methyl Ester	Exposure (Days)	Tritium Incorporated (Mc.)	Distribution of Tritium, %			
			Unsaponifiable	Labile	Acid aqueous	Fatty acids
Oleate	18	8.3	14.6	7.7	1.2	76.5
Linoleate	18	14.4	12.9	5.3	0.3	81.5

Methyl oleate. The results of gas chromatography and subsequent liquid scintillation counting of methyl oleate immediately after tritium irradiation are given in Fig. 1. This pattern is similar to that for the saturated ester[6] in showing a small amount of rapidly eluted radioactive material coincident with the solvent peak which is thought to be either residual tritium gas or solvent molecules containing labile tritium. However, the pattern differs from that for the saturated esters in that the major radiochemical peak is eluted prior to the inactive oleate peak. Similar behavior of the major radiochemical component is shown in Fig. 2 for the liquid-partition chromatogram of the oleic acid preparation after saponification, exchange of labile

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(5) K. E. Wilzbach, *J. Am. Chem. Soc.*, **79**, 1013 (1957).

(6) R. F. Nystrom, L. H. Mason, E. P. Jones, and H. J. Dutton, *J. Am. Oil Chemists' Soc.*, **36**, 212 (1959).

(7) H. J. Dutton and R. F. Nystrom, Proceedings of the Symposium on Advances in Tracer Applications of Tritium, New York, N. Y., October 31, 1958, pp. 8–15.

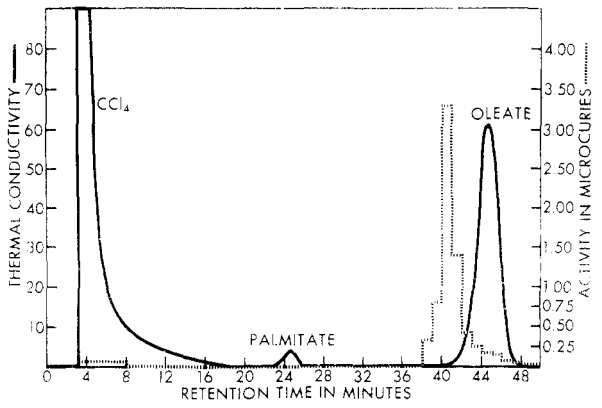


Fig. 1. Gas chromatogram of methyl oleate immediately after exposure to tritium gas

tritium, and acidification. Moreover, this assay technique could be used as a preparative method for isolating radiochemically pure methyl tritiostearate (56 curies per millimole for the addition of T_2 (pure) to C=C). It will become apparent that this shift is not an isotopic effect;[8] rather, the radiochemical peaks in Figs. 1 and 2 are coincident with the positions of methyl stearate and stearic acid, respectively, and the radioactive methyl stearate is produced by the saturation of the double bond in methyl oleate with tritium.

After chemical purification and methylation of the oleic acid by diazomethane (Fig. 3), the effectiveness of our purification procedures can be demonstrated by gas chromatography. The gas analysis shows that labile tritium is absent in the solvent peak, confirms the shift in position of the radiochemical compound compared to inactive methyl oleate, and establishes that the radiochemical product behaves chemically and chromatographically as a methyl ester of stearic acid. In further experiments, the coincidence of this radiochemical peak with that for methyl stearate was established by adding inactive methyl stearate to the tritiated methyl oleate sample.

On periodate-permanganate oxidation of the chemically purified tritiated oleic acid, the acids were separated on a liquid-partition column designed for separating monobasic acids as one peak from the separate peaks of the C_{10}, C_9, and C_9 dibasic acids. Two major peaks corresponding to monobasic acids and azelaic acid were obtained. In Fig. 4 radiochemical activity is coincident with the peak for unresolved monobasic acids and the azelaic acid is inactive.

Subsequent resolution of the monobasic peak on a liquid-partition chromatographic column designed specifically to separate the expected monobasic acids shows (Fig. 5) that the pelargonic acid is also inactive and that the radiochemical activity is coincident with titratable amounts of stearic acid coming from a known methyl stearate impurity

(8) K. E. Wilzbach and P. Riesz, *Science*, 26, 748 (1957).

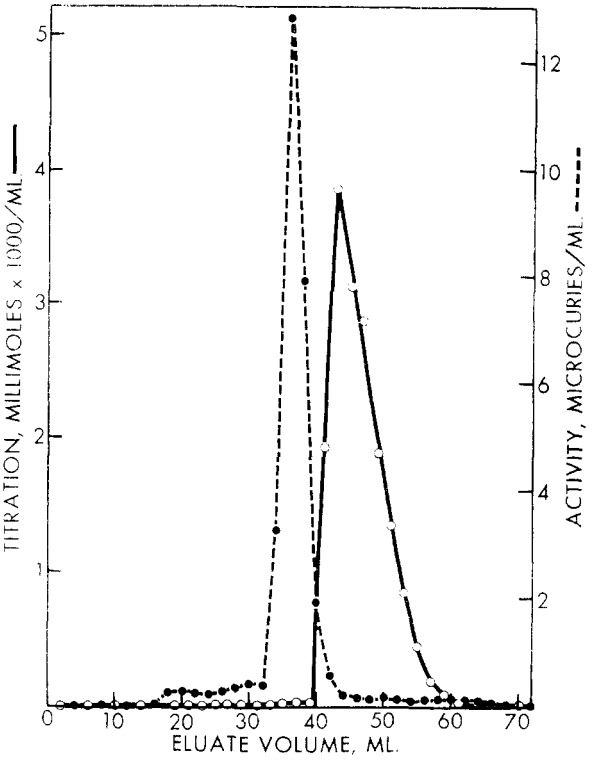
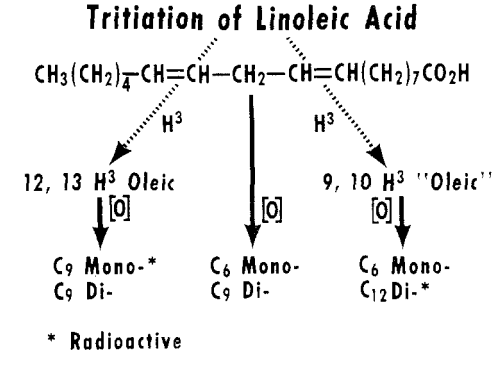


Fig. 2. Liquid-partition chromatogram of a mixture of chemically purified tritiated oleic acid and carrier oleic acid

in the original untritiated methyl oleate preparation. The radioactivity of this stearic acid and the inactivity of the pelargonic and azelaic acids were confirmed (not shown) by gas chromatography of the mixture of their methyl esters and by radiochemical assay of the fractions.

Methyl linoleate. Gas-chromatographic data for the tritiated methyl linoleate before (Fig. 6) chemical purification show the displacement of the radioactive peak from the inactive parent methyl linoleate to the position expected for methyl oleate. The coincidence of positions for the tritiated linoleate with methyl oleate was confirmed by gas chromatography of a mixture of inactive methyl oleate and tritiated methyl linoleate and by observing coincidence of peaks.

The addition of tritium to methyl linoleate can occur at two positions: the 9,10-double bond and the



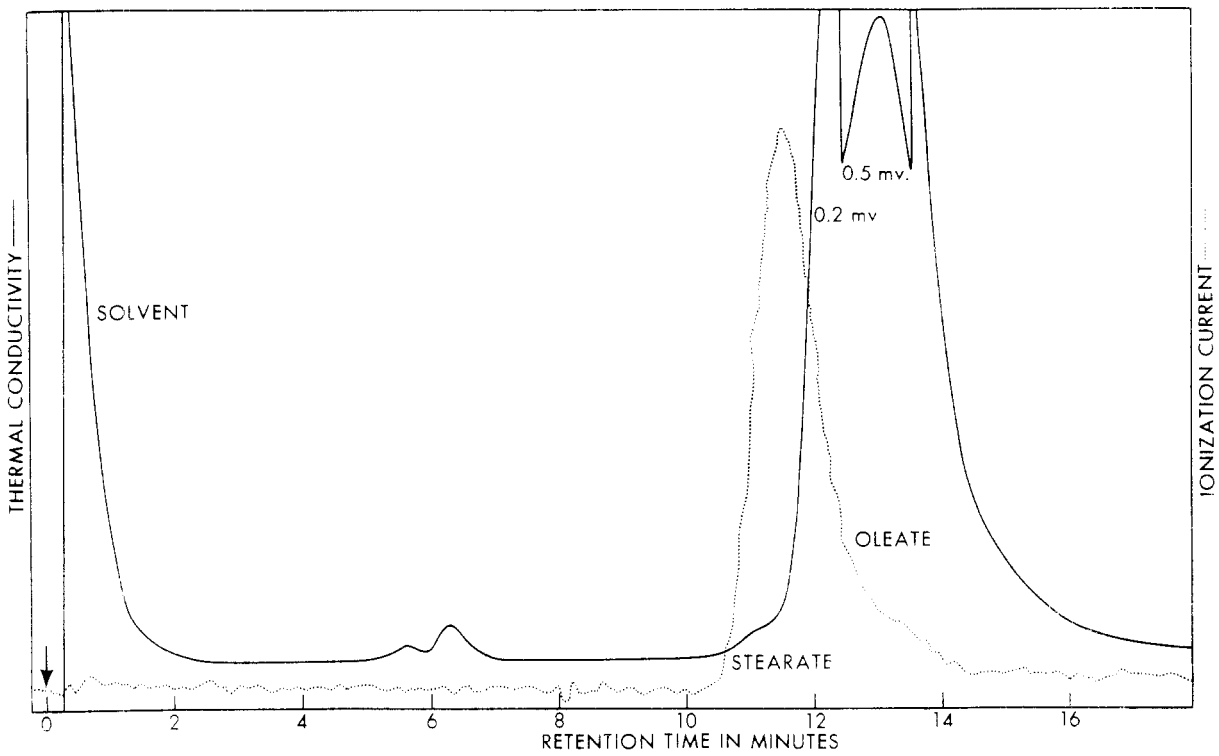


Fig. 3. Gas chromatogram of tritiated methyl oleate after saponification, alcohol exchange, acidification, and methylation

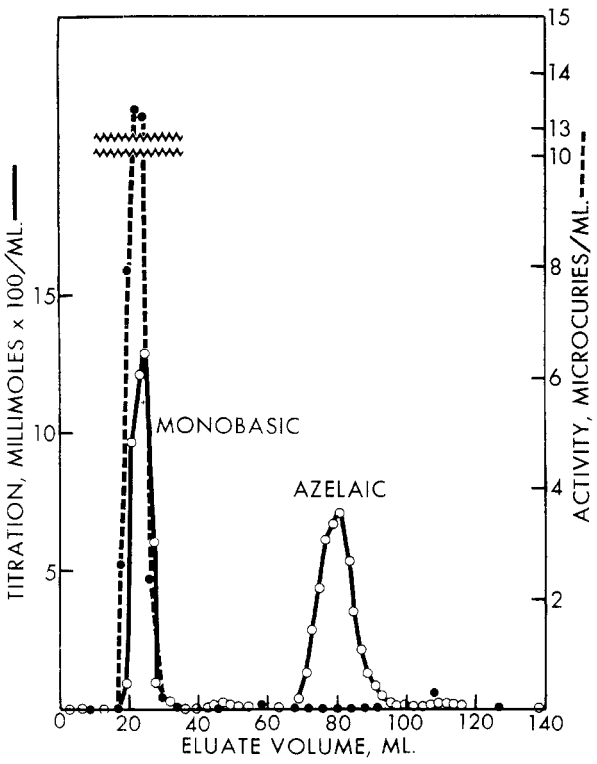


Fig. 4. Partition-chromatogram of mono- and dibasic acids from tritiated oleic acid after oxidative cleavage

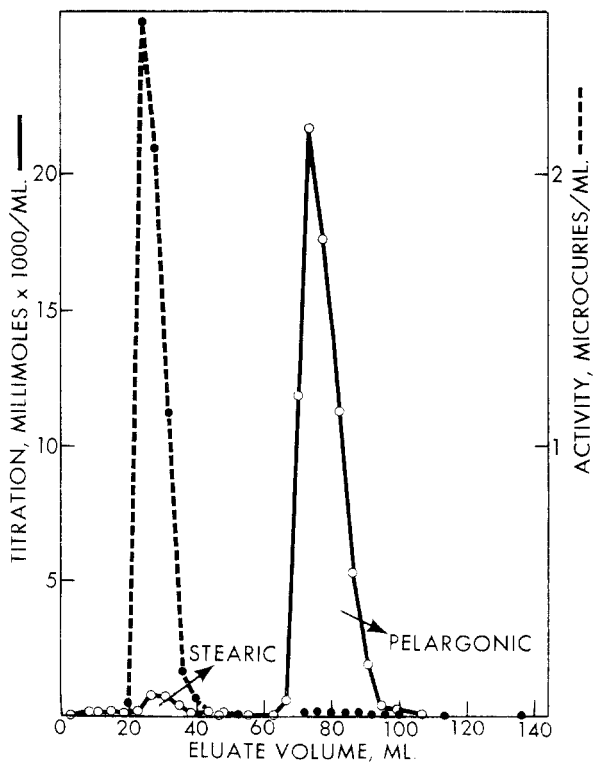


Fig. 5. Liquid-partition chromatogram of the monobasic peak of Fig. 4

12,13-double bond. After oxidation of tritiated methyl linoleate, subanalytical amounts of radioactive pelargonic and radioactive dodecanedioic acid are anticipated. The major constituents expected from the inactive linoleic acid are caproic and azelaic acids. (Malonic acid is not eluted from the chromatogram used.)

Confirmation of the radioactivity of the C_{12} di-

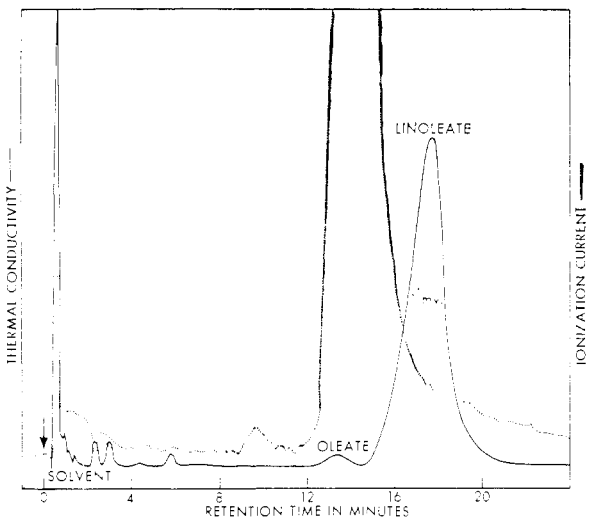


Fig. 6. Gas chromatogram of methyl linoleate after exposure to tritium gas but before chemical purification

basic (dodecanedioic acid) and the C_9 monobasic (pelargonic) acids, as well as the inactivity of the major constituents, caproic and azelaic acids, is found in Figs. 7 and 8. Fig. 7 shows that all radioactivity is in the region of the titrated monobasic peak, and no activity is present in the azelaic or suberic acid peaks; the latter is found in the acidic mixture probably because of the presence of an ester, unsaturated at the C_8 position, in the original methyl linoleate. Similar and confirmatory data by gas chromatography were obtained but are not presented. To separate the two activity peaks associated with the titratable monobasic peak, a

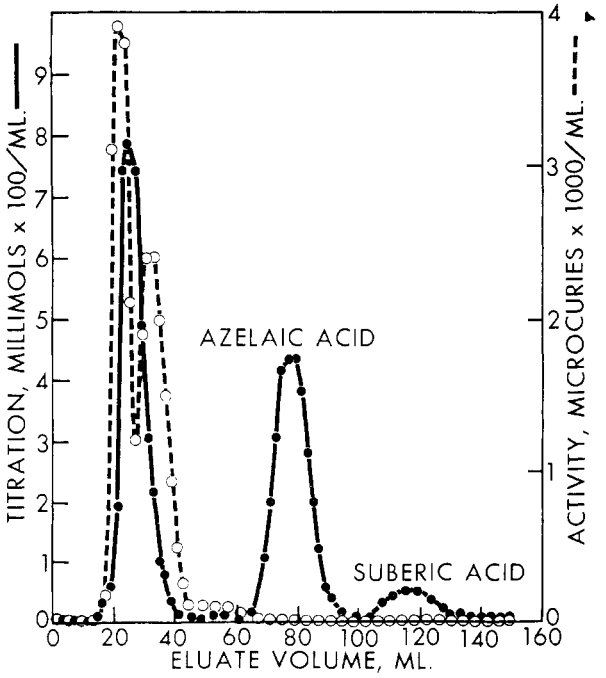


Fig. 7. Liquid-partition chromatograms of the mono- and dibasic acids from tritiated linoleic acid after oxidative cleavage

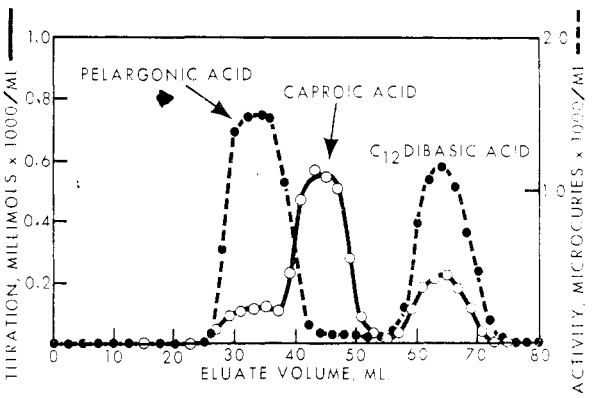


Fig. 8. Liquid-partition chromatogram showing the resolution of the monobasic peak of Fig. 7 (added pelargonic acid and dodecanedioic acids)

special column was designed for better separation of these two acids. In the chromatogram shown in Fig. 8, small amounts of pelargonic and dodecanedioic acids were added to the monobasic acids (peak of Fig. 7) for identification purposes. The chromatogram shows that the monobasic acid fraction is indeed composed of active pelargonic acid, inactive caproic acid, and active dodecanedioic acid. Estimates of the relative activity in the pelargonic and the C_{12} dibasic acids lead to the conclusion that the 12,13 unsaturated carbons are the preferred position of attack by tritium over the 9,10 carbons by a ratio of about 1.4:1.0.

DISCUSSION

The results of the investigations demonstrate the need for extreme care in establishing the structure of radiochemical products formed by the technique of gas exposure to tritium. The conclusions suggest that addition of tritium may be the generalized or usual reaction for olefinic compounds. It is of considerable interest that exposure of methyl linoleate did not result in the formation of methyl stearate but only one of the two double bonds was reduced.

Separation by chromatography of the radiochemical products from their inactive isologous parent results in isolation of fatty acids of extremely high specific activities. If the addition comprises one hydrogen and one tritium atom, the activity of the labeled product amounts to 28 curies per millimole.

Although the main object of this research was to produce or prepare randomly and substitutively labeled unsaturated fatty acids, it has not been achieved. Instead, labeled octadecanoates and octadecenoates were formed with high specific activities, and they may have many uses where the number and not the position of double bonds is important.

EXPERIMENTAL

Tritiation of methyl oleate. One gram of methyl oleate (Hormel Institute) and 1 curie of tritium gas were placed

in a glass tube 25 mm. in diameter and 65 mm. long. This ampoule was rotated to provide continuously renewed thin films of the ester. After 18 days, the excess tritium was removed and the residue was refluxed for 1 hr. with 0.6 ml. of 50% sodium hydroxide diluted with 10 ml. of ethanol. On addition of 25 ml. of water, the unsaponifiable material was removed by extraction with five successive 25-ml. portions of ether. Removal of labile tritium was accomplished by distillation of 1.5 l. of anhydrous ethanol from the soaps in 50-ml. batches. Finally, 9 ml. of 1*N* hydrochloric acid was added and the organic acids extracted with four successive 25-ml. portions of ether.

Gas chromatography of tritiated methyl oleate. Methyl oleate after exposure to tritium gas as well as after tritiation, saponification, alcohol exchange, acidification, and methylation (with diazomethane) was examined for chemical and radiochemical purity by gas chromatography on a 5-ft. Resoflex 296 column at 205° in the "Aerograph" instrument.[9] Simultaneously with the recording of thermal conductivity, an ion chamber–electrometer system recorded radioactivity (ion current) on the gas stream issuing from the thermal conductivity cell.[10] Alternatively radioactivity was determined by trapping the methyl esters in the effluent gas stream in vials containing 15 ml. of scintillation solution for minute intervals and by subsequent assay in the automatic "Tri-Carb Scintillation Spectrometer."[9]

Liquid partition chromatography of tritiated acids. Chemical and radiochemical purity of tritiated fatty acids (after alcohol exchange, *etc.*) was determined by the liquid-liquid partition chromatographic procedure of Nijkamp[11] which employs a methanol-isooctane solvent system on a silicic acid column. Alternate 1-ml. eluate fractions were (a) titrated in a nitrogen atmosphere with 0.2*N* potassium hydroxide to a thymol blue end-point using a Gilmont Microburet, and (b) diluted with 15 ml. of scintillation solution for assay of radioactivity with an automatic "Tri-Carb Scintillation Spectrometer." Quenching of fluorescence by fatty acids and by the chromatographic solvent was negligible.

Degradation of tritiated esters. Oxidative cleavage of the double bonds in the tritiated esters was accomplished by the method of Jones and Stolp[12] after addition of the inactive ester to the tritiated ester. This method involved saponification of the ester, oxidation of the acids as soaps at room temperature with periodate-permanganate solution, and subsequent extraction of all ether-soluble acids in a continuous extractor. Only traces of radioactivity remained in the aqueous layer. Removal of ether gave the acids for chromatographic identification.

Liquid-liquid partition chromatography of monobasic and dibasic acids from methyl oleate. Upper and lower phases of a water-alcohol-benzene mixture at equilibrium comprised the mobile and immobile phases, respectively. Silicic acid was used as the solid support.[12] All monobasic acids emerge as one peak and dibasic acids appear as separate peaks. Alternate 1-ml. eluate fractions were titrated and assayed with the scintillating spectrometer. Monobasic fractions were combined, acidified, and extracted. The acids were identified by the chromatographic procedure of Nijkamp,[1] titrated, and assayed for radioactivity.

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(9) Since the Department of Agriculture does not recommend the products of one company over those of another, the names are furnished for information only.

(10) L. H. Mason, H. J. Dutton, and L. Bair, *J. Chromatog.*, **2**, 322 (1959).

(11) H. J. Nijkamp, *Anal. Chim. Acta.*, **10**, 448 (1954).

(12) E. P. Jones and J. A. Stolp, *J. Am. Oil Chemists, Soc.*, **35**, 71 (1958).

[CONTRIBUTION FROM THE NAVAL STORES RESEARCH STATION[1]

Esters of Some Acids Derived from Terpenes[2]

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Received December 28, 1959

The preparation of vinyl monomers for use as internal plasticizers for polyvinyl chloride from acids derived from terpenes led to the preparation of six new vinyl esters. Acylpinolic acids were prepared by reaction of pinolic acid with the respective anhydrides and direct reaction with the acids in the presence of an acid catalyst. From the substituted pinolic acids, pinolic acid, pinonic acid, and 3-(1-methyl-1-hydroxyethyl)heptanedioic acid γ-lactone, the new vinyl esters were prepared by vinyl interchange with vinyl acetate in the presence of mercuric sulfate catalyst. Ethyl esters of these acids were prepared by azeotropic removal of water from the reaction mixture with *p*-toluenesulfonic acid as catalyst. The pinonic and pinolic ethyl and propyl esters have been previously reported. The identity of the ethyl esters obtained by direct esterification and those obtained by catalytic reduction of the vinyl esters was established by means of infrared analyses.

The propyl and allyl esters of pinolic and pinonic acids were prepared by direct esterification in the presence of *p*-toluenesulfonic acid. Dehydration and rearrangement of pinolic acid in the presence of *p*-toluenesulfonic acid was observed but not reported in detail.

In the course of investigating terpene derived materials for use in the preparation of polymerizable monomers of interest as internal plasticizers for polyvinyl chloride, vinyl esters were prepared from a number of terpene acids and derivatives of these acids. Two allyl esters were also prepared. For comparison purposes the corresponding ethyl and propyl esters were prepared by reduction of the unsaturated esters and by direct esterification.

The acids involved in this work were pinonic [3 - acetyl - 2,2 - dimethylcyclobutaneacetic acid), pinolic [3-(1-hydroxyethyl)-2,2-dimethylcyclobutaneacetic acid], pinolic acid acetate, pinolic acid propionate, pinolic acid butyrate, 3-(1-methyl-1-hydroxyethyl)heptanedioic acid γ-lactone, pinic acid [3-carboxy-2,2-dimethylcyclobutaneacetic acid], and several monoalkyl pinates. The esters

(1) One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented at the meeting of the American Chemical Society, Atlantic City, New Jersey, September 13–18, 1959.