sensitivity. By condensing in a scintillation solution, the radioactivity may be subsequently observed at the convenience of the operator and for sufficiently long periods of time to obtain the desired statistical results.

REFERENCES

1. Arnoff, S., "Techniques of Radiobiochemistry," The Iowa State College Press, Ames, Iowa, (1957).
2. Dutton, H. J., Jones, E. P., Mason, L. H., and Nystrom, R. F., Chem. and Ind., No. *36*, 1176–1177 (Sept. 6, 1958).
3. Jones, E. P., Mason, L. H., Dutton, H. J., and Nystrom, R. F., J. Org. Chem., *25*, 1413–1417 (1960).
4. Nijkamp, H. J., Anal. Chim. Acta, *10*, 448–458 (1954).
5. Kerr, V. N., Hayes, F. N., and Ott, D. G., Intern. J. of Appl. Radiation and Isotopes, *1*, 284–288, (1957).
6. Schram, E., and Lombaert, R., Anal. Chim. Acta, *17*, 417–422 (1957).
7. Funt, B. L., and Hetherington, A., Science, *129*, 1429–1430 (1959).
8. Bangham, D. R., Biochem. J., *62*, 552–553 (1956).
9. Greenfield, S., The Analyst, *83*, 114–116 (1958).
10. Steinberg, D., Nature, *182*, 740–741 (1958).
11. Dutton, H. J., Jones, E. P., Scholfield, C. R., Chorney, W., and Scully, N. J., J. Lipid Research, *2*, 63–67 (1961).
12. Dutton, H. J., Scholfield, C. R., and Mounts, T. L., J. Am. Oil Chemists' Soc., *38*, 96–101 (1961).
13. Dutton, H. J., Jones, E. P., Nystrom, R. F., J. Org. Chem., in press.
14. Dutton, H. J., Scholfield, C. R., Jones, E. P., Chem. and Ind., in press.
15. Karmen, A., and Tritch, H. R., Nature, *186*, 150–151 (1960).
16. Dutton, H. J., ms. in preparation.
17. Popjak, G., Lowe, A. E., Moore, D., Brown, L., and Smith, F. A., J. Lipid Research, *1*, 29–39 (1959).
18. Emmett, P. H., Abstract of Papers, 135th Meeting, Am. Chem. Soc., Boston, Mass., April 5–10, 1959, p. 3Q.
19. Wolfgang, R., and Rowland, F. S., Anal. Chem., *30*, 903–906 (1958).
20. James, A. T., and Piper, E. A., J. Chromatog., *5*, 265 (1961).
21. Riesz, P., and Wilzbach, K. E., J. Phys. Chem., *62*, 6–9 (1958).
22. Mason, L. H., Dutton, H. J., Bair, L. R., J. Chromatog., *2*, 322–323 (1959).
23. Cacace, F., and Inam-Ul-Haq., Science, *131*, 732–733 (1960).
24. Dobbs, H. E., J. Chromatog., *5*, 32–37 (1961).

Fatty Acid Structure Determination by Chemical Means

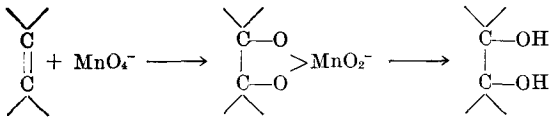
ROBERT A. STEIN, Department of Physiological Chemistry, University of California, Los Angeles, California

THE UTOPIAN GOAL for a chemist performing a structure determination is to possess an unknown compound in a high state of purity and to command reactions that proceed in an unambiguous manner. In practice, a compromise with either of these ideals still makes it possible to adequately determine a structure, but the lack of both of them will severely cloud the interpretation of the data.

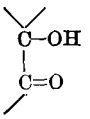
The various chromatographic techniques are effective in preparing pure samples of fatty acids that are initially contaminated with homologues or dissimilar compounds, but greater stress is placed on such techniques in separating closely allied isomers. Obvious examples of these are the isomeric unsaturated fatty acids (including geometric as well as positional isomers) and positional isomers of branched chain acids. The extent to which small quantities of these isomeric compounds can be found depends on the specificity of the chemical reagents used in the structure determination. For this reason, this discussion will consider the qualitative nature and quantitative limitations of some reactions useful to the lipid chemist in solving identification problems not easily solved by chromatographic techniques alone. Further, consideration will be limited to the fatty acids with a hydrocarbon chain and will not include those with other functional groups.

Potassium permanganate is useful in cleaving fatty acids at centers of unsaturation. An examination of the mechanism and experimental conditions used with this reagent will assist in understanding the limitations or sources of difficulty in using it.

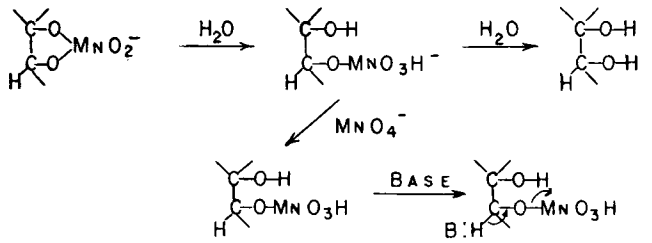
A simplified view of a thorough permanganate oxidation of a double bond shows the products as two carboxylic acids with a concomitant reduction of the Mn(VII). The reaction however is not simple, and inevitably other products are formed; the course of the reaction is determined by pH conditions. An investigation of the oxidation of oleic acid using O^{18}-labeled permanganate (1) in alkaline solution confirmed the theory that the first step of the oxidation involved the formation of a cyclic ester between the olefin and permanganate ion which was followed by hydrolysis in the alkaline solution to the glycol. The recovery of high yields of the glycol indicates it is stable toward oxidation under these conditions.



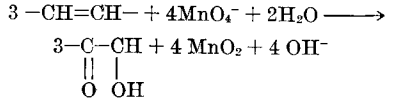
Oxidation under less alkaline conditions results in a further oxidation of the olefin to the ketol or acyloin (2).



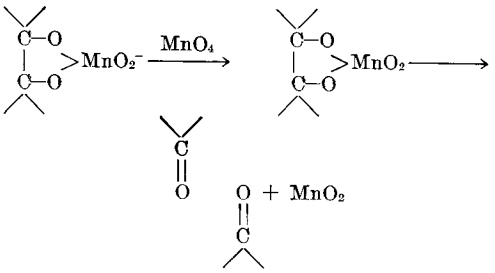
Conditions (pH 9) that give the ketol in good yields (75%) from oleic acid do not oxidize the glycol to the ketol (3). To reconcile this apparent anomaly, it is considered (1) that the cyclic ester is a common intermediate in both reactions and undergoes rapid hydrolysis to the half ester. At high pH, hydrolysis is complete, resulting in glycol formation, while in somewhat less basic medium the ketol formation involves a further oxidation of the Mn(V) in the ester which then undergoes a concerted elimination reaction with base.



One consequence of the oxidation of an olefin with potassium permanganate is that the solution becomes alkaline during the reaction and thus may change its course (2). As the pH increases the major product being formed changes from ketol to glycol.



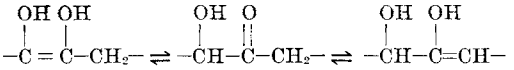
Under neutral or acid conditions, the course of the oxidation changes and results in carbon-carbon bond cleavage. By one proposed mechanism, further oxidation of the first-formed cyclic ester intermediate yields a neutral ester which cleaves to the two aldehydes. These subsequently are oxidized to the corresponding acids (1).



Since neutral or acid conditions for the permanganate oxidation results in olefin splitting, these are the conditions most useful in obtaining fragments for a structure determination. The double bond cleavage however does not give the corresponding carboxylic acids free from other products (4). Chromatographic evidence indicates that the oxidation of various unsaturated acids in acetic acid produces a series of dicarboxylic acids shorter than that expected. To further investigate the extent of this overoxidation and the effect of the method of addition and type of solvent, oleic acid was oxidized with permanganate under varying conditions (5).

In general the oxidations were performed on 0.1 mM of oleic acid with 1 mM of $KMnO_4$ in 0.5 ml of solvent at 15–21°C. When the solvent was acetic acid the amount of C_8 dicarboxylic acid formed with respect to the expected C_9 was consistently between 3 and 5%. The addition of fluoride ion, as KF, which is known to inhibit the oxidation of oxalic and malonic acids by acid permanganate (6) had no effect on the extent of overoxidation. Other overoxidation products include C_7 dicarboxylic acid (10–20% of the C_8 diacid) and an unknown compound in lower concentration that has a trivial coincidence with the methyl ester of C_{11} dicarboxylic acid on a polyester gas column. The relative amounts of these extra oxidation products are independent of order of addition of the reactants (e.g., solid $KMnO_4$ to the oleic acid in solvent or the oleic acid to the $KMnO_4$ in solvent). The type of solvent used however changes the relative yield of C_8 and C_9 diacids. In acetone and butanone the amount of C_8 increased to 8–14%. In pentane and benzene very little oxidation occurred, but that which was oxidized gave nearly equal quantities of C_8 and C_9 diacids.

From these data obtained in nonpolar solvents it seems evident that the formation of the lower chain homologues does not occur by oxidation of the carboxylic acids once they are formed, but rather at some intermediate stage. In the oxidation of ketones by other oxidants it has been shown that the rate of oxidation is dependent upon the rate of enolization of the ketone. In the present examples of the overoxidation of monoenoic fatty acids, it is probable that the extent of overoxidation is being determined by a rate or extent of enolization of an intermediate compound prior to further oxidation. This intermediate might be a ketol, for example, which can enolize in two ways.



Further oxidation of the ketol or the enol on the left would result in the expected cleavage products, while oxidation of the other enol would ultimately result in an acid one carbon shorter than expected.

The oxidation of stearolic acid gives evidence that the overoxidation can occur before the chain is broken. Cleavage of the triple bond gives acids without an intervening aldehyde. Since a homologous series of acids is formed as overoxidation products during the oxidation of stearolic acid, and since the carboxylic acids themselves are not oxidized under the conditions used, it seems clear that the overoxidation started before the chain was broken.

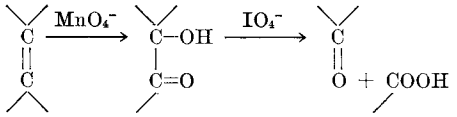
However the oxidized but still intact chain is not the only source of overoxidation products. Oxidation of fatty aldehydes by permanganate in acetic acid gives a homologous series of fatty acids shorter than the anticipated product (5). It is expected that the stepwise degradation of the aldehyde occurs by oxidation of the enol to form a new aldehyde one carbon shorter, thereby completing one turn of the degradation spiral.

With a judicious choice of conditions, permanganate cleavage of olefins to carboxylic acids is a satisfactory method for structure determination; but because of the formation of small quantities of extra oxidation products, its use in determining the small amounts of closely allied isomers (e.g., 5% Δ^8 C_{18} acid in oleic acid) would be unsatisfactory. The practical use of permanganate oxidations is also complicated by the necessity of isolating the products from the reaction mixture. As each product is extracted from the reaction mixture, it will be distributed in the solvents according to its particular partition coefficient. Since this coefficient is different for each product, the extract will not be quantitatively representative of the original products until the extraction is complete.

Acetylene bonds are also cleaved by permanganate oxidation, the reaction being pH dependent. Stearolic acid at pH 7 is oxidized in 92–96% yield to 9,10-dioxooctadecanoic acid. At a higher or lower pH no diketone is isolated and the expected carboxylic acids are obtained (9). Since these cleavage fragments are identical to those obtained from an ethylene bond in the same position, permanganate oxidation would not

be the reagent of choice to differentiate between double and triple bonds.

An interesting adjunct to double bond cleavage by permanganate is the use of a catalytic amount of permanganate with an excess of periodate (7). The permanganate oxidizes the olefin to a glycol or ketol and the periodate effects both the cleavage and the regeneration of permanganate. The aldehydes are oxidized to the acids by the permanganate.



The reported yield of the expected dicarboxylic acid from the oxidation of unsaturated acids is within 2% of the expected values, but again one major drawback is the quantitative recovery of the products from the dilute solutions.

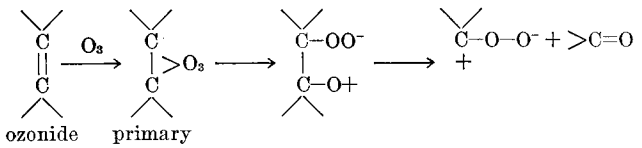
An interesting speculation on the permanganate-periodate oxidation is that the periodate is not the obligatory glycol-splitting oxidant in the reaction, but that its function is to maintain a low concentration of permanganate. If this is the case, the periodate could be replaced by another oxidant capable of oxidizing manganese dioxide to permanganate.

For a more detailed account of the oxidations with permanganate, attention is directed toward two recent reviews (8).

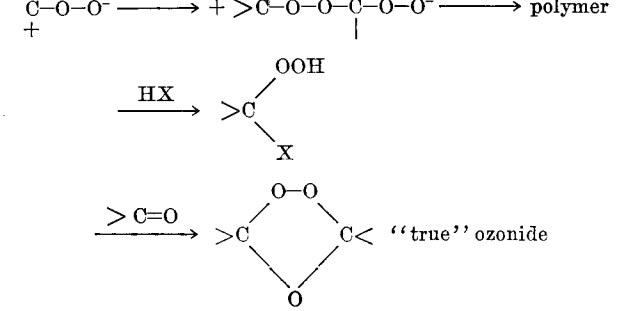
Ozonolysis

Ozonolysis is an alternative to other oxidative means of multiple bond cleavage. In conjunction with sensitive chromatographic means of identifying the products it becomes a simple, fast, quantitative process substantially free from side reactions. Reviews of the reaction of ozone with organic compounds document the excellent research that revealed the mechanisms of these reactions (10).

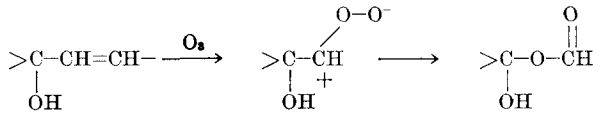
Ozone, acting as an electrophilic reagent, adds to an olefin to form a primary ozonide, which in turn decomposes to an aldehyde (or ketone) plus a zwitterion. There is some evidence that the primary ozonide is stable below −60°C. but undergoes changes above this temperature (11).



The zwitterion is capable of polymerizing, rearranging, reacting with an HX solvent (X = OH, OR, OCOR), and adding to aldehydes and ketones to form the "true" ozonide.



The zwitterion rearrangement occurs when there is an allylic substituent such as hydroxy, ether or amino in the original olefin.



Awareness of the possibility of such rearrangements will prevent misinterpretation of data obtained from some hydroxylated unsaturated fatty acids.

With the exception of the rearranged compound, the ozonolysis products give the expected carboxylic acids and/or ketones on oxidation, and aldehydes and/or ketones on mild reduction. Both techniques have been used in obtaining products from unsaturated fatty acids. Oxidation of ozonized oleic acid by molecular oxygen gives only moderate yields of dicarboxylic acid (12), while silver oxide, even after a prolonged reaction time of three days, did not completely oxidize all of the aldehydes to the corresponding acids (4d). Hydrogen peroxide and performic acid are also unsatisfactory in giving clean oxidation products (4b).

Reduction of ozonides to the aldehydes and/or ketones, on the other hand, can give pure products and avoids the necessity for esterification before gas column analysis. No attempt will be made here to review all of the reducing agents that have been used, and this discussion will consider only a catalytic hydrogenation and one chemical method that are known to give superior results with unsaturated fatty acids.

In brief, when hydrogenation is chosen for the reduction, a suitable method is as follows (13): a few milligrams of the sample to be oxidized in methylene chloride or methanol at −60°C are allowed to react with ozone (3% in O_2) until a faint blue color develops in the solution. Oxygen and ozone are then removed from the solution by a nitrogen purge. Hydrogen is next bubbled through at room temperature, the addition of Lindlar catalyst affecting the desired reduction within 30 min. Removal of the catalyst leaves a solution of neutral products that may be analyzed directly by gas chromatography.

The chemical reduction method is attractive because of its even greater simplicity (14). The ozonized sample in methylene chloride or other solvent, after being flushed with nitrogen, is reacted at the ozonization temperature with triphenyl phosphine. The phosphine may be added in the solid form or as a solution. The reaction solution is allowed to warm to room temperature and a sample is injected directly into a gas chromatographic column.

The phosphine reduces the ozonide and is itself oxidized to the corresponding phosphine oxide (15).

Unlike the catalytic hydrogenation method, which results in a chemically purer reaction product, the chemically reduced samples contain the phosphine and phosphine oxide. If the analysis is performed by gas phase chromatography on a polyester column the triphenyl phosphine is eluted with a relative

retention time of approximately 10 with reference to methyl stearate; no evidence that the phosphine oxide is eluted has been obtained. The contaminating phosphorus compounds thus do not normally interfere in the chromatography.

In order to examine both the short chain aldehydes and the aldehyde esters obtained from the usual unsaturated fatty esters, it has been found convenient to perform the ozonolysis in different solvents and to analyze the product on polyester columns at different temperatures. Methylene chloride allows an examination of the aldehyde esters but obscures the more volatile simple aldehydes. For these, pure methyl octanoate is a good solvent, as it allows the chromatography of more volatile aldehydes before they are swamped by the solvent peak. Ozonolysis in methyl octanoate requires a higher temperature because of the decreased solubility of some of the samples in this ester, but the −20°C temperature at which it may be used does not affect the character of the products.

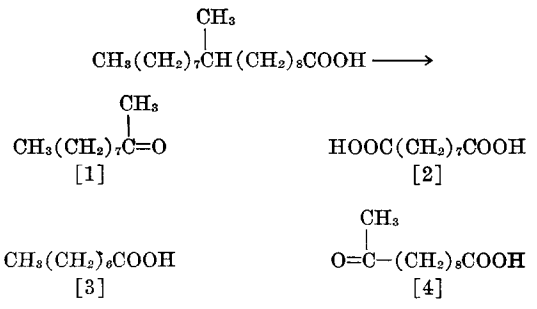
The cleavage products obtained from oleic, linoleic, linolenic, and arachidonic methyl esters were obtained free from contaminating products and semiquantitatively. Malonaldehyde is exceptional in that its observed response with an argon detector is less than expected.

The ozonolysis of acetylene bonds may occur in two stages. On ozonolysis, stearolic acid yields the expected carboxylic acids and in addition a 4% yield of 9,10-dioxooctadecanoic acid (9). This diketone is formed by a partial cleavage of the triple bond. As the acetylene bond does not give an aldehyde on ozonolysis, this method is useful in determining the positions of double and triple bonds in the same molecule (16). Acetylenes are also less reactive than ethylenes and, under controlled conditions, a molecule containing both types of unsaturation may be cleaved at the double bond only, thus leaving the acetylene intact (17).

Determination of Position of Methyl Branches

A problem considerably more refractory than that of the position of unsaturation is locating the position of a methyl substituent of the fatty acid chain. With sufficient material and patience the entire molecule could be degraded one carbon at a time until the substituents were found; but this being impractical, except for substituents close to the carboxyl group, alternative methods must be sought.

One such alternative method is the chromic acid oxidation and cleavage at the site of branching (18).



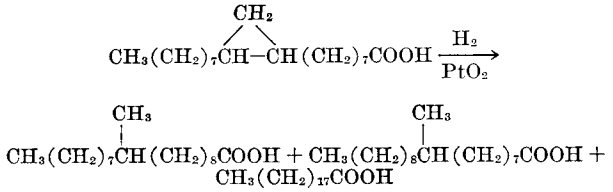
For example, when the oxidation of 300 mg of 10-methylstearic acid occurs in acetic acid for 2–3 hours at 60–70°C and with a 6:1 ratio of chromium trioxide to fatty acid, the yield of products 1, 2, 3, and 4 are, respectively, 1, 22, 10, and 9%. A preliminary separation of the ketone from the acidic products revealed the expected ketone to be the primary neutral product. Gas chromatography of both the neutral and the acidic material (after conversion to the methyl esters) may be expected to yield sufficient information for an unequivocal structure determination. With conversion to isolable products so low however the ability to identify small amounts of positional isomers in a mixture might be seriously limited.

The presence of the methyl substituent at positions 2, 3, 4, and 5 decreases the yield of identifiable products and, in the case of 3- and 4-methyl, none of the expected products are formed. This apparent change in reactivity as the site of action approaches the carboxyl group needs further clarification, but presently suggests caution in interpreting results from compounds with suspected branches near the carboxyl group.

Methods are available for the introduction of a tertiary hydroxy group at the site of branching in fatty acids. Yields of 50–90% are obtained with alkaline permanganate or manganate (19). Exploiting this substitution with subsequent chain cleaving reactions should give an attractive micromethod for determining branched chain positions.

Cyclic Fatty Acids

Fatty acids with a carbocyclic structure in the chain are exemplified by sterculic and chaulmoogric acids. The sterculic acid contains a cyclopropene structure (20) and chaulmoogric a cyclopentene ring (21). In both acids the double bonds behave as normal olefins on oxidation with permanganate or ozone. A difference however is found between the reactivity of the dihydro (saturated) compounds with respect to catalytic hydrogenation. The five-membered ring is unaffected, while the cyclopropane structure of dihydrosterculic acid is opened by hydrogenation (Adams catalyst) to three compounds: two with methyl branches and one with a normal chain (20).

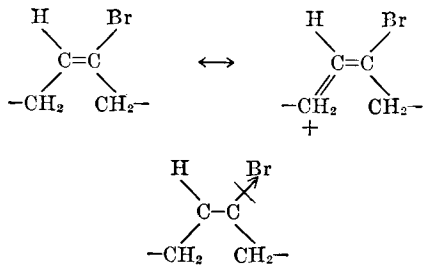


The problems posed by carbocyclic systems in fatty acids may thus be reduced to those that have been dealt with above: namely, the interpretation of oxidation data obtained from multiple bond cleavage and the location of chain branching, whether it be in a carbocyclic ring or as a methyl group.

Determination of Cis-Trans Isomers

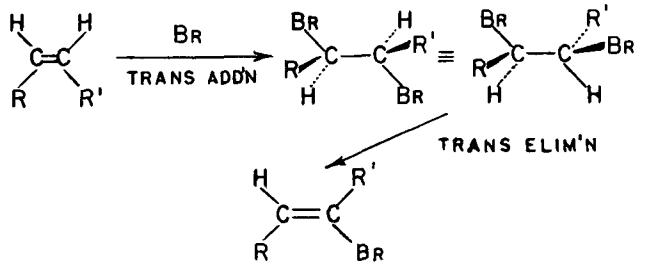
Despite the great sensitivity of gas column chromatography for some types of structural features, the identification of *cis* and *trans* isomers is not possible with the usual polyester liquid phases that are widely used for fatty ester analyses. It may be argued that the small differences in dipole moment between the *cis* and *trans* configuration is insufficient to permit separation, and hence that incorporation of a dipole-moment-enchancing substituent might well make such differentiation possible.

Studies of gas column chromatography of 9- or 10-bromo-substituted methyl oleate and elaidate justify this working hypothesis (22). The vinyl substituted oleate is expected to have a higher dipole moment than the corresponding elaidate because of a resonance contribution having a dipole oriented in the same direction as that of the C-Br dipole (23).



This difference between the *cis* and *trans* compounds allows a separation on a polyester gas column with the less polar *trans* or elaidate preceding the more polar *cis* or oleate.

Experimentally the monoenoic acids are readily converted into the vinyl bromides *via* two stereospecific reactions (24).



A *cis* olefin is stereospecificity converted into the *threo* dibromide, which dehydrobrominates in the presence of base to yield the *trans* olefin with a vinyl bromine substituent. Conversely, the *trans* isomer yields a product having *cis* geometry.

The utility of these transformations in determining *cis* and *trans* isomers has been demonstrated with mixtures of oleic and elaidic acids (22). Thirty mg. of fatty acids, better than 99% pure, were brominated and dehydrobrominated with sodium methoxide, then esterified with diazomethane, and chromatographed on a polyester column. Oleic acid containing 5.9% elaidic by weight gave a chromatographic analysis of 5.3%, while elaidic acid containing 2.5% oleic gave 4.5%.

REFERENCES

1. Wiberg, K.B., and Saegebarth, K.A., J. Am. Chem. Soc., *79*, 2822 (1957).
2. Coleman, J.E., Ricciuti, C., and Swern, D., J. Am. Chem. Soc., *78*, 5342 (1956).
3. King, R., J. Chem. Soc., 1788 (1936).
4. Bergemann, P.H., Kepplar, S.G., and Boekenoogen, H.A., Rec. trav. chim., *69*, 439 (1950); (b) James, A.T., and Webb, J., Biochem. J., *66*, 515 (1957); (c) Fulco, A.J., and Mead, J.F., J. Biol. Chem., *234*, 1411 (1959); (d) Cason, J., and Tavs, P., *ibid.*, *234*, 401 (1959).
5. Stein, R.A., and Slawson, V., unpublished.
6. Launer, H.F., and Yost, D.M., J. Am. Chem. Soc., *56*, 2571 (1934); Drummond, A.Y., and Waters, W.A., J. Chem. Soc., 2456 (1954).
7. Lemieux, R.U., and von Rudloff, E., Can. J. Chem., *33*, 1701 (1955); von Rudloff, E., J. Am. Oil Chemists' Soc., *33*, 126 (1956).
8. Ladburg, J.W., and Cullis, C.F., Chem. Rev., *58*, 403 (1958); Waters, W.A., Quart. Rev., *12*, 277 (1958).
9. Kahn, N.A., and Newman, M.S., J. Org. Chem., *17*, 1063 (1952).
10. Criegee, R., Record Chem. Progr., *18*, 111 (1957); Bailey, P.S., Chem. Rev., *58*, 925 (1958).
11. Criegee, R., Abs. 136th Meeting Am. Chem. Soc., 87P (1959).
12. Naudet, M., and Pasero, J., Fette u. Seifen, *62*, 1110 (1960).
13. Privett, O.S., and Blank, M.L., J. Lipid Res., *2*, 37 (1961).
14. Stein, R.A., and Nicolaides, N., unpublished.
15. Horner, L., and Hoffmann, H., Angew. Chem., *68*, 473 (1956).
16. Hatt, H.H., and Szumer, A.Z., Chem. and Ind., 962 (1954).
17. Bohlmann, F., and Sinn, H., Chem. Ber., *88*, 1869 (1955).
18. Cason, J., Fessenden, J.S., and Agre, C.L., Tetrahedron, *1*, 289 (1959).
19. Kenyon, J., and Symons, M.C.R., J. Chem. Soc., 2129 (1953); *ibid.*, 3580 (1953).
20. Nunn, J.R., J. Chem. Soc., 313 (1952).
21. Shriner, R.L., and Adams, R., J. Am. Chem. Soc., *47*, 2727 (1925).
22. Stein, R.A., unpublished.
23. Hannay, N.B., and Smyth, C.P., J. Am. Chem. Soc., *68*, 1005 (1946).
24. Hoff, M.C., Greenlee, K.W., and Board, C.E., J. Am. Chem. Soc., *73*, 3329 (1951).