[CONTRIBUTION FROM THE FRUIT PRODUCTS DIVISION, UNIVERSITY OF CALIFORNIA]

Acid Metabolism of Wine Yeast. I. The Relation of Volatile Acid Formation to Alcoholic Fermentation

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It has been shown definitely that the volatile acids invariably found among the products of yeast fermentation of nutrient media containing fermentable sugars are formed by the yeast[2,3] and not by the non-enzymatic oxidation of alcohol as claimed by several early investigators.[4]

As a result of the investigations of Kostytchev[5] and Neuberg[6] the occurrence of acetic acid is commonly ascribed to intermolecular oxidation–reduction of acetaldehyde. The occurrence of formic acid[7] has not been explained as adequately.[8] The yeasts used by Kostytchev and by Neuberg apparently did not form any formic acid under the conditions of their experiments.[9] It is possible that the formic acid is formed by yeast from pyruvic acid,[10] as is believed by some to be the case for bacterial metabolism. Gvaladze[2] concluded, from a quantitative determination of the by-products of alcoholic fermentation of grape juice or cane sugar mineral salts media, that all the volatile acid formed could be accounted for as acetic acid formed according to Neuberg's third form of fermentation.

If we ascribe the origin of volatile acid to the diversion of a part of the intermediate product of alcoholic fermentation, acetaldehyde, from its normal path (hydrogenation to alcohol) and its subsequent transformation by intermolecular oxidation–reduction to acetic acid and alcohol, it is difficult to explain why this diversion should occur, as found by Reisch[2] and confirmed by Osterwalder[2] and ourselves, largely during the initial stages of the fermentation and why it practically ceases after the initial sugar content has been reduced in half (see Figs. 1 and 2). Our investigations indicate that most of the volatile acid formed during the normal fermentation is produced during the period of rapidly decreasing oxidation–reduction potential (see Fig. 3). However, there is no direct relation between volatile acid production and oxidation–reduction potential when fermentations are conducted in the presence of various gases.

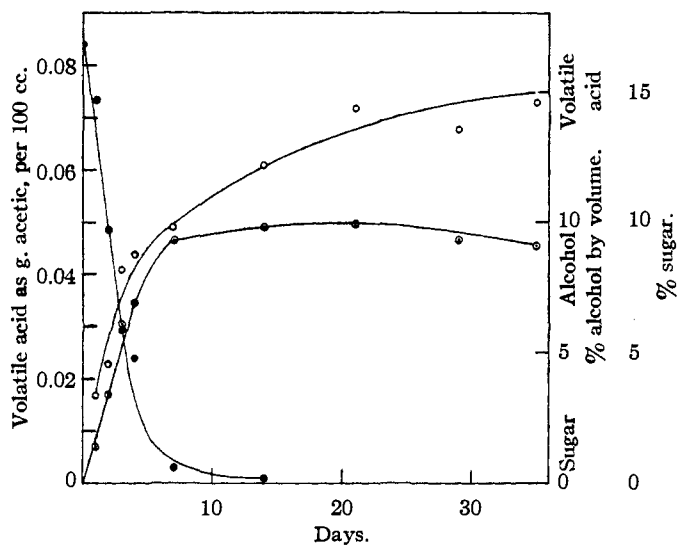

Fig. 1.—Formation of volatile acids by Burgundy yeast. Fermentations conducted in 500-cc. cotton-stoppered bottles containing 250 cc. of sterile filtered white grape juice. Two or three bottles were removed at each sampling period. The initial acidity of the juice was 0.34 as g. of tartaric acid per 100 cc. and *p*H 3.94. Volatile acidity continued to increase up to about 56 days (0.092) and began to decrease thereafter until it reached 0.016 at 282 days.

(1) Worker on Works Progress Administration Project No. 5456, under which this investigation was carried out.

(2) Arminus Bau in Franz Lafar, "Handbuch der technischen Mykologie." Vol. IV, 1905–1907, pp. 384–386; V. Gvaladze, "Correlation Among the Products of Alcoholic Fermentation," Lenine Agr. Acad. U. S. S. R., 1936 (in Russian with French summary); L. Pasteur, *Compt. rend.*, **48**, 1149 (1859); E. Maumené, *ibid.*, **57**, 398 (1863); A. Béchamp, *ibid.*, **75**, 1036 (1872); E. Prior, *Bayer Brauer J.*, **5**, 49 (1895); E. Kayser, *Ann. Inst. Pasteur*, **10**, 51 (1896); U. Gayon, *Rev. vit.*, **7**, 461 (1897); W. Seifert, *Centr. Bakt.*, Abt. 2, **3**, 337 (1897); R. Reisch, *ibid.*, Abt. 2, **14**, 572 (1905); A. Osterwalder, *ibid.*, Abt. 2, **32**, 481 (1912); R. Meissner, *Z. Gärungsphysiol.*, **2**, 129 (1913).

(3) E. Buchner and J. Meisenheimer, *Z. physiol. Chem.*, **40**, 167 (1903); *Ber.*, **37**, 417 (1904); **38**, 620 (1905).

(4) A. Trillat, *Compt. rend.*, **146**, 645 (1908); R. Duchemin and J. Dourlen, *ibid.*, **140**, 1466 (1905); L. Mathieu, *Bull. assoc. chim. sucr. dist.*, **22**, 1283 (1905).

(5) S. Kostytchev, *Z. physiol. Chem.*, **89**, 367 (1914).

(6) C. Neuberg and J. Hirsch, *Biochem. Z.*, **96**, 175 (1919); **100**, 304 (1919).

(7) Khondabachian, *Ann. Inst. Pasteur*, **6**, 600 (1892); P. Thomas, *Compt. rend.*, **137**, 1015 (1903); P. Esau, Univ. Calif. Master's Thesis in Bacteriology, 1933.

(8) For example, see M. Schoen, "The Problem of Fermentation," Chapman Hall Ltd., London, 1928, pp. 62–67.

(9) S. Kostytchev and L. Frey, *Z. physiol. Chem.*, **146**, 276 (1925).

(10) C. Neuberg, *Biochem. Z.*, **67** 90–101 (1914).

Experimental Procedure

A non-agglomerating wine yeast, yeast 66, isolated from California grapes by W. V. Cruess in 1911[11] and subsequently used in a series of investigations in this Laboratory,[12] and Burgundy yeast, an agglomerating yeast originally imported from France for use in California wineries,[13] were used in these tests.

The fermentations in the preliminary tests were conducted in tall 8-liter bottles, filled with about 6 liters of sterile grape juice, which were fitted with gas inlet tubes, sampling tubes closed off with two-way stopcocks, two platinum wire electrodes, and a potassium chloride-agar bridge. Samples of about 100 cc. were withdrawn periodically for analysis. The fermentations were carried out at room temperature, 24–27°, 1% by volume of an active twenty-four or forty-eight hour starter being used.

In the oxidation–reduction experiments the fermentations were conducted in rubber-stoppered 800-cc. jars filled with about 500–600 cc. of sterile juice and fitted with two platinum-wire electrodes, potassium chloride-agar bridge, sampling tube and gas inlet tube. Samples of about 15 cc. were withdrawn periodically for analysis. These jars were placed in an oil-bath maintained at 30 ± 0.1°.

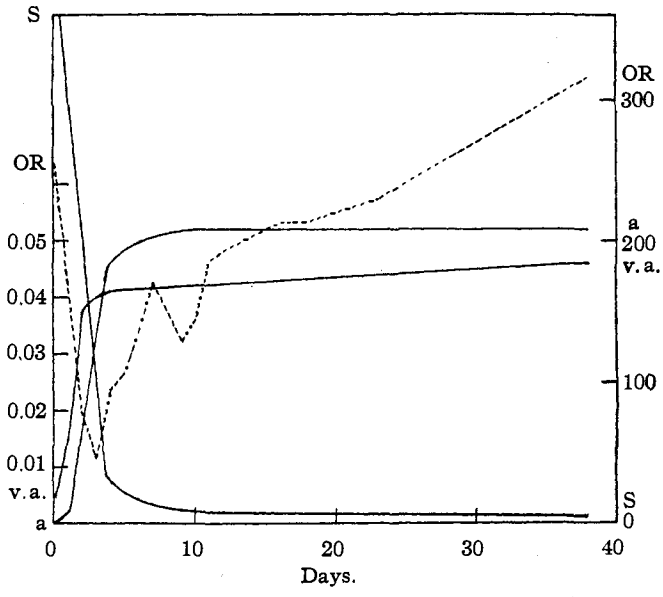

Fig. 2.—Volatile acid formation as related to course of fermentation by yeast 66 in grape juice. The initial total acidity of the juice was 0.415, *p*H 3.7.

Sterile compressed air, oxygen, nitrogen and carbon dioxide, respectively, were passed through the medium for a period of about twelve hours before inoculation. The flow of gas was not measured but as estimated from a count of the rate of bubbling it was approximately 75–100 cc. of gas per minute.

The alcohol content was determined by ebullioscope; the volatile acidity was determined by titrating in the hot 100 cc. of steam distillate from 10-cc. sample; the sugar content was determined by the ferricyanide procedure of Hanes[14] but using the reagents of Blish and Sandstedt[15] and is expressed as grams dextrose per 100 cc. The volatile acids for the red juice were fractionated by the steam distillation procedure of Dyer[16] after colorimetric tests[17] had shown that only formic and acetic acids were present.

The oxidation–reduction potential was determined by measuring the potential of platinum wire electrodes against a saturated calomel cell using an L. and N. guarded millivolt potentiometer No. 7652 and an L-N type R reflecting galvanometer No. 2500-e. A 2 megohm resistance was placed in series with the electrodes to reduce polarization during measurements and this was shorted to 1 megohm as the e. m. f. was balanced. The air and oxygen fermentations were poorly poised in comparison with the others.

Data and Discussion

Formation of Volatile Acids in Normal Fermentations.—It is evident from the data summarized in Fig. 1 that the first rapid increase in volatile acid content by Burgundy yeast occurred during the stage of active fermentation, resulting in a decrease of sugar content from 16.7 to about 5.0. A further increase occurred during the storage period, reaching a value of over two times that formed in fermentation. In later stages a decrease in volatile acidity occurred. The total acid content changed in about the same way, the *p*H value decreasing about 0.2 during active fermentation and remaining approximately constant thereafter. The changes in volatile acid content are affected by differences in strain of yeast and experimental conditions (*cf.* Figs. 1 and 2 with data given by Reisch[2]). The results obtained in large-scale fermentations in practice indicate but slight increase in volatile acid content after approximately half the sugar has been fermented (Zimmermann[18]). The theory that the formation of volatile acids during the active stages of fermentation is due to the dehydrogenation of acetaldehyde is strengthened by the more rapid accumulation of glycerol during the same period, according to the data obtained by Seifert and Reisch.[19] The increase in volatile acidity

(11) W. V. Cruess, *Univ. Calif. Pub. Agr. Sci.*, **4**, 1–66 (1918).

(12) H. Aref and W. V. Cruess, *J. Bact.*, **27**, 443 (1934); R. L. Tracy, *ibid.*, **24**, 423 (1932).

(13) F. T. Bioletti, *Calif. Agr. Expt. Sta. Circ.*, **23**, (1906); F. T. Bioletti and W. V. Cruess, *Calif. Agr. Expt. Sta. Bull.*, **230**, 64–86 (1912).

(14) C. S. Hanes, *Biochem. J.*, **23**, 99 (1929).

(15) M. J. Blish and R. M. Sandstedt, *Cereal Chem.*, **10**, 189 (1933).

(16) D. C. Dyer, *J. Biol. Chem.*, **28**, 445 (1916).

(17) Henri Agulhon, *Bull. soc. chem.*, [4] **9**, 881 (1911).

(18) J. G. Zimmerman, *Wein Rebe*, **18**, 352 (1937).

(19) W. Seifert and R. Reisch, *Centr. Bakt.*, Abt. 2, **12**, 574 (1904).

following fermentation is probably due to the oxidation of alcohol by yeast.[20] The decrease in volatile acid content noted in samples stored in the presence of air is probably due to the dehydrogenation of acetic acid.

As shown in Fig. 2, the rapid accumulation of volatile acid by yeast 66 occurred also during the period of rapid fermentation during which a decrease in E_h occurred. Very little volatile acid was formed during the period in which the E_h remained stationary or during the period in which it increased. It is of course possible that acid formation and acid destructive processes might be occurring at practically equal rates.

Effect of Aeration on the Formation of Volatile Acids by Wine Yeast.—The changes occurring during fermentation of 6 liters of freshly prepared white grape juice and diluted white grape concentrate, respectively, with and without aeration were compared. Compressed air was bubbled through the media during the first two weeks of the test and was then turned off. The data obtained, of which Fig. 3 is typical, indicate that although the course of alcoholic fermentation was not greatly affected by aeration, volatile acid formation by both Burgundy and 66 yeast was depressed markedly during the initial stages, although subsequently the volatile acidity of the aerated samples rapidly increased to a value higher than that for the unaerated ones.

Effect of Oxidation-Reduction Potential on Volatile Acid Formation.—It is well known that the oxidation-reduction potential of a sugar solution undergoing alcoholic fermentation by yeast rapidly decreases to a certain low level, believed to be characteristic of the fermentation process, and remains at that level during the duration of the normal fermentation.[21] Now if the volatile acids are formed during the period of decreasing oxidation-reduction, their formation should be influenced by any change in rate of decrease of oxidation-reduction or by any artificial poising of the oxidation-reduction potential. To determine the possibility of poising the oxidation-reduction potential of a fermenting solution by bubbling through it gases of different oxygen tension and to obtain more detailed information on volatile acid formation, several tests were made with diluted white grape concentrate and white grape juice fermented in the presence of air, oxygen, nitrogen and carbon dioxide, at 30°. The results for typical experiments are shown in Figs. 4 and 5. It is, of course, obviously impossible to duplicate closely the conditions of any one test,[21b] particularly as regards oxidation-reduction potentials. However, the results of all tests show quite definitely that, for white grape juice, with the onset of fermentation the oxidation-reduction potentials drop quite rapidly to a level varying from −140 to −180 mv. (E_h of 100 to 60 mv.) under our conditions even in presence of air or oxygen which initially increase the oxidation-reduction potential. The length of time the solution remains at the low level depends on the oxygen concentration of the gas, varying from five to ten hours for pure oxygen and from fifteen to thirty hours for air. Carbon dioxide gas was found to be more efficient than nitrogen in preventing a rise in oxidation-reduction potential, probably as a result of absorption of oxygen from the air. The increase in oxidation-reduction potential was faster for oxygen than air and the fermentation solution was finally poised at an oxidation-reduction level considerably higher than the original.

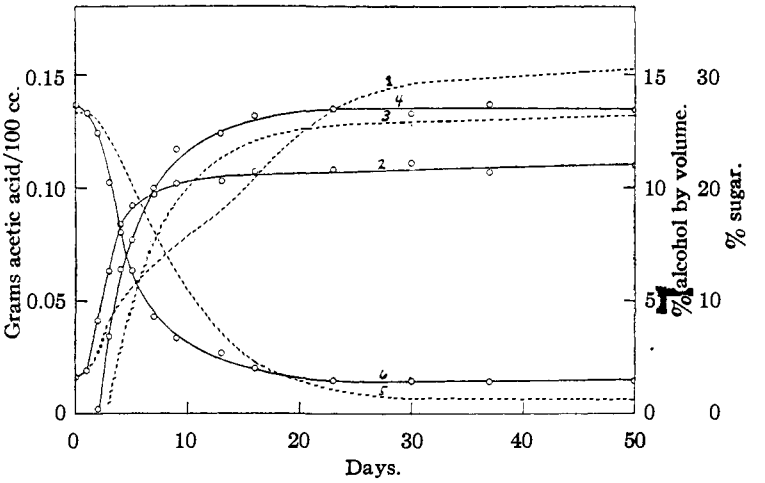

Fig. 3.—Volatile acid production by 66 in diluted grape concentrate, with and without aeration for the first eleven days: 1, volatile acid (aerated); 2, volatile acid (plain); 3, alcohol (aerated); 4, alcohol (plain); 5, sugar (aerated); 6, sugar (plain).

The volatile acidity of the samples fermented

(20) Robert Sonderhoff, *Ergebnisse Enzymforschung*, **3**, 163 (1934).

(21) (a) J. C. Hoogerheide, "Contribution to the Knowledge of the Pasteur Reaction," Doctor's Thesis, Delft, 1935; (b) A. J. Kluyver and J. C. Hoogerheide, *Biochem. Z.*, **272**, 197 (1934); (c) A. J. Kluyver and J. C. Hoogerheide, *Enzymologia*, **1**, 1 (1936).

in presence of oxygen increased as the oxidation–reduction decreased, then remained practically constant until the oxidation–reduction increased when the volatile acidity increased also. The initial increase was in general less than that of the untreated samples but the subsequent increase was much greater. The volatile acidity of the samples fermented under aeration, with one exception, was lower than and remained lower than the check. There is some evidence for destruction of volatile acids in the presence of air rather than their formation by oxidation. It is again likely that both processes occur. In the presence of carbon dioxide, more volatile acid is formed than in the presence of nitrogen, even though the latter maintains a lower oxidation–reduction level. Carbon dioxide retarded the fermentation more than did nitrogen and where this retardation was marked there also was a more noticeable production of volatile acids.

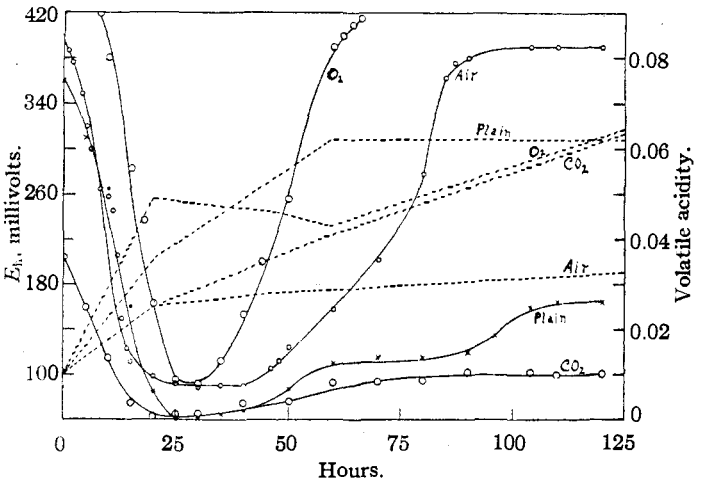

Fig. 4.—Rate of change of E_h in white grape juice, and the corresponding volatile acid formation (initial *p*H 4.10, final *p*H, 3.8–4.0). The volatile acidities for oxygen, air, CO_2, N_2 and plain samples, respectively, after 20 hours were 0.049, 0.025, 0.025, 0.033, 0.036; after 68 hours—0.043, 0.029, 0.041, 0.050, 0.062; after 224 hours—0.100, 0.046, 0.071, 0.049, 0.062. In three runs with diluted grape concentrate the initial order was O_2, CO_2, N_2, P, air; the final order after over 200 hours was O_2, CO_2, P, N_2 and air.

Similar trends were found in red grape juice, the increase in volatile acid content of the juice fermented under oxygen or air during the period of decreasing oxidation–reduction being less than for the others, but subsequently the volatile acid in both cases was higher. The data indicate somewhat closer correlation between the trends in volatile acid production and in oxidation–reduction potential than for white juice although in either case there is no quantitative relation between the two.

The volatile acids present in the final sample were analyzed with the results shown in Table I. Formic acid apparently forms a large proportion of the volatile acid content (*cf.* Esau[9]). There is some evidence for an increase in formic acid formation in presence of oxygen but the data for nitrogen and carbon dioxide are somewhat conflicting.

Summary and Conclusions

1. The formation of volatile acids by wine yeasts during the alcoholic fermentation of grape juice was studied in relation to the changes in alcohol and sugar content and to changes in oxidation–reduction potential. The effects of fermentations with oxygen, air, nitrogen and carbon dioxide gases were compared.

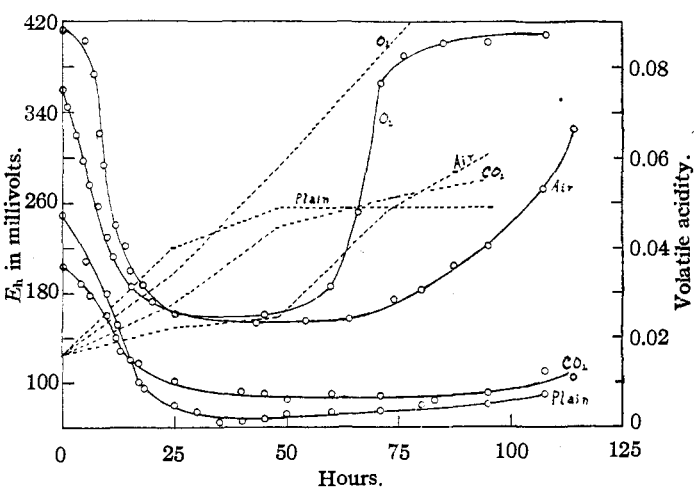

Fig. 5.—Rate of change of E_h and corresponding volatile acid formation in red grape juice (initial *p*H 3.92, final *p*H 3.5–3.8). After 24 hours the volatile acid content of samples in presence of O_2, air, CO_2, N_2 and plain, respectively, was: 0.031, 0.023, 0.026, 0.025 and 0.026; after 72 hours—0.071, 0.057, 0.049, 0.040, 0.046; and after 178 hours, 0.153, 0.106, 0.080, 0.066 and 0.055. Duplicate runs agreed in the trends, that initially the order of decreasing volatile acid formation was oxygen, nitrogen, plain, CO_2 and air, whereas finally it was oxygen, air, CO_2, N_2 and plain.

2. Volatile acids were found to be formed

TABLE I

VOLATILE ACIDS FORMED IN RED WINE

Gas	Run 1, g./100 Formic	Run 1, g./100 Acetic	Run 2, g./100 Formic	Run 2, g./100 Acetic
None	0.0226	0.0327	0.0289	0.0292
Air	.0325	.0514	.0489	.0686
Oxygen	.0275	.090	.0273	.132
Nitrogen	....	....	.0162	.0532
Carbon dioxide	.0388	.0186	.0308	.0492

largely in the initial stages of the fermentation when the fermenting medium was protected from oxidation. In the presence of oxygen, volatile acids may be formed or utilized depending on experimental conditions.

3. The formation of volatile acids during the active stage of fermentation corresponds to the stage of decreasing oxidation–reduction potentials.

4. It is impossible to poise the oxidation–reduction potentials of fermenting grape juice by the use of oxygen or air although both reduce the period of time during which the oxidation–reduction level is maintained at the low level characteristic of fermentation and both increase the subsequent rise in oxidation–reduction potential.

5. In grape juice fermented under aeration less volatile acid is formed in the initial stages than in carbon dioxide, nitrogen or plain. Oxygen exerts a similar but not so marked effect and for a shorter period.

6. The changes in volatile acid production are ascribed to the activity of the yeast dehydrogenases in oxidizing aldehydes, alcohol and acetic acid, respectively. It is suggested that there is an association or competitive action on all three substrates. However, these investigations do not exclude the possibility that substances such as fixed acids, tartaric acid, malic acid or N-constituents may not be involved.

7. The role and origin of formic acid found among the volatile acids in fermented juice is yet to be explained.

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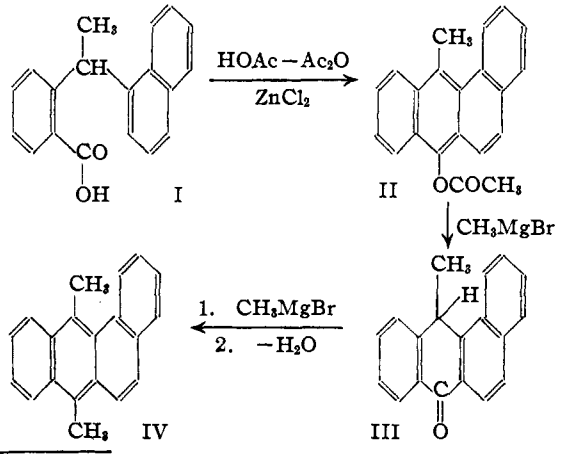
The Synthesis of 9,10-Dimethyl-1,2-benzanthracene

BY MELVIN S. NEWMAN

Because of the high degree of cancer producing activity of 10-methyl-,[1] 5,10-dimethyl-,[1] and 5,9-dimethyl-1,2-benzanthracene,[2] it seemed of interest to synthesize 9,10-dimethyl-1,2-benzanthracene.

Work was commenced upon the most direct approach, namely, the addition of two moles of methylmagnesium bromide to 1,2-benzanthraquinone, but discontinued upon the appearance of the article of Bachmann and Bradbury,[3] who advised us that they were continuing this line of work with the intention of synthesizing 9,10-dimethyl-1,2-benzanthracene. However, as material was desired for biological work, it was deemed worth while to attempt the preparation of this hydrocarbon by a different method.

The starting material for the presently reported synthesis was *o*-(α-methyl-α-1-naphthyl)-toluic acid[2,4] I, which was cyclized to 10-acetoxy-9-methyl-1,2-benzanthracene, II, and the latter compound hydrolyzed to the anthrone, III, by the method of Fieser and Hershberg.[5] The anthrone (or its tautomeric isomer) was not isolated in a pure condition as it could not be crystallized. The final hydrocarbon, IV, obtained by the reaction of III with methylmagnesium bromide followed by dehydration of the resulting carbinol proved identical with the 9,10-dimethyl-1,2-benzanthracene synthesized by



(1) Fieser and Newman, THIS JOURNAL, **58**, 2376 (1936).
(2) Newman, *ibid.*, **59**, 1003 (1937).
(3) Bachmann and Bradbury, *J. Org. Chem.*, **2**, 175 (1937).
(4) Cook, Robinson, and Goulden, *J. Chem. Soc.*, 393 (1937).
(5) Fieser and Hershberg, THIS JOURNAL, **59**, 1028 (1937).