1516

The Composition of Honey. V. Separation and Identification of the Organic Acids

EDGAR E. STINSON, MARY H. SUBERS, JEANNE PETTY AND JONATHAN W. WHITE, JR.

The Composition of Honey. V. Separation and Identification of the Organic Acids[1]

EDGAR E. STINSON, MARY H. SUBERS, JEANNE PETTY AND JONATHAN W. WHITE, JR.

From the Eastern Regional Research Laboratory,[2] Philadelphia, Pennsylvania

The acids from clover honey were isolated by ion-exchange adsorption and separated by silicic acid partition chromatography and ion-exchange chromatography. The different acids were identified by paper chromatography with six solvent systems, infrared spectra of the sodium salts, and derivatives. Acids definitely identified were butyric, acetic, formic, lactic, succinic, pyroglutamic, malic, citric, and gluconic. Oxalic acid was tentatively identified.

Gluconic acid, not citric acid as previously proposed, was the principal acid of honey.

INTRODUCTION

As part of a program conducted in this laboratory on the composition and properties of honey, an investigation was made of the acidic materials in Eastern clover honey. A number of early investigators, including Farnsteiner (1) and Fincke (2), investigated the reported preponderance of formic acid in honey and concluded that this acid actually contributes less than 10 % to the acidity of honey. Heiduschka (3) reported finding butyric, valeric, caproic, capric, malic, lactic, and oxalic acids, basing the identifications on nonspecific precipitation and oxidation methods. Several of the volatile acids were "identified" by odor. Nelson and Mottern (4) isolated citric, malic, and succinic acids from a number of honeys and identified them by crystallography and melting points of derivatives. The occurrence of calcium oxalate in honey was reported by Philipsborn (5). Vavruch (6) identified malic, tartaric, citric, lactic, and succinic acids by paper chromatography. Angeletti (7) reported the presence of gluconic acid in samples of musty honey, but this acid has not been reported in normal honey. Goldschmidt and Burkert (8) used lead-salt precipitation of the acids extracted by ether from honey followed by paper chromatography. They identified acetic, formic, citric, malic, and maleic acids. Citric and malic were converted to derivatives; crystalline maleic acid was isolated and identified by melting point and conversion to fumaric acid. Enzymic production of keto acids was suggested by Cocker (9) to explain the apparent increase in acid material during alkali titration which had been observed by Walton (10). White, Petty, and Hager (11) were unable to repeat the heat inactivation reported by Cocker, which was the basis of his enzyme postulation, and attributed the apparent increase in acid content to the presence of lactone material. The possibility of enzymic action, detected by other means, was not eliminated. They analyzed 225 samples of honey using a rapid titration procedure designed to distinguish between free acids and lactone and

[1] Presented before the Division of Agricultural and Food Chemistry at the 136th national meeting of the American Chemical Society, Atlantic City, N. J., Sept. 16, 1959.

[2] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

found the lactone content to be considerable, often being half as great as the free acid content.

It was the purpose of this investigation to establish definitely the identity of the major acids found in honey, preparing derivatives whenever possible, and to identify the lactone material.

MATERIAL AND METHODS

The honey examined was white Eastern clover honey of characteristic flavor which had been pasteurized by the ordinary commercial procedure and had no visible evidence of fermentation.

The acids were separated from the honey by ion-exchange resins and then fractionated on silicic acid and ion-exchange columns. The use of these two chromatographic methods based on different principles of operation permitted comparison of the results. For example, gluconic acid was very difficult to elute from the silicic acid column but was easily removed from the ion-exchange column.

SILICIC ACID CHROMATOGRAPHY

Several preliminary separations of honey acids were made using the 8-g. silicic acid column described by Bulen, Varner, and Burrell (12). Alternate effluent fractions were titrated, and the effluent diagrams resulting were virtually identical with that shown in Fig. 1. Rechromatography of fractions frequently resulted in unexplained losses of materials, though yields of knowns from the original column were generally quantitative. Substitution of 0.01 *N* HCl for 0.05 *N* H_2SO_4 in preparing the columns resulted in more symmetric elution curves and better paper chromatograms. This modification was used in preparing subsequent silicic acid columns.

Large-Scale Silicic Acid Chromatography

A quantity of 3190 g. honey was diluted to 20% solids, preserved by toluene, and the inorganic cations and amino acids were removed by passing the solution through a column containing 125 ml. Dowex 50,[3] 50–100 mesh. The acids were then adsorbed by passing the effluent solution immediately through a column containing 125 ml. Duolite A-2, 50–100 mesh, at approximately 12 ml./min.

[3] Mention in this article of commercial products or equipment under names of their manufacturers does not constitute endorsement by the U. S. Department of Agriculture of such products, equipment, or firms.

As previously noted with the preliminary experiments, acidity began to appear in the eluate after only 1500 ml. of the honey solution had passed through the column. (Hydrolysis of neutral lactone material?) The entire honey solution was passed through the columns which were rinsed with distilled water until the eluate gave a negative anthrone test for carbohydrate. The Duolite column was then stripped of acids with 440 ml. of 1 *N* NaOH. The cations were removed from the solution by an acidic cation-exchange column, and the effluent was neutralized with alkali using a potentiometer.

Acidity of the Original Honey and of the Various Fractions

Original honey:	
By back titration	80 meq.
By direct titration	51.5
Difference	28.5 meq.
Column fractions:	
I Passed through columns, not retained	47.8 meq.
II Washed by water from Duolite column	11.9
III Retained on Duolite column and eluted	49.2
	108.9 meq.

A portion of I equivalent to 675 g. original honey was again passed through the ion-exchange system. The effluent showed no acidity. After washing, the material was eluted from the Duolite column, titrated, and freeze-dried. An equivalent weight of 191 was found; theory for gluconic acid is 196. Paper chromatography indicated, among other materials, gluconic acid. No further work was done on this material.

The apparent increase in acidity was assumed to be due to the removal of cations originally present in the honey and possibly enzyme action. Only III was examined in detail; 41.5 meq. III, 7 ml. 6 *N* HCl, and 14 g. dry silicic acid were mixed and added to a 160-g. silicic acid column (60 cm. × 3.8 cm. O.D.). Fractions of 6.6 ml. were collected and every tenth or twentieth titrated; results are plotted in Fig. 1.

The fractions comprising the various peaks were combined and neutralized, and the solutions were evaporated to dryness. The residues were redissolved, acidified with cation-exchange resin, and examined by paper chromatography. Solvents used for this preliminary examination were those of Kennedy and Barker (13) (volatile acids) and

n-butanol–acetic acid–water (14) (nonvolatile acids).

On a later confirmatory experiment, this procedure was repeated using steam distillation to remove the volatile acids from the concentrated acid solution. Both the volatile and the nonvolatile acids were chromatographed on silicic acid columns which were developed with chloroform solutions containing 1 and 3% *n*-butanol (15) for separation of the volatile acids before using higher concentrations of butanol.

Ion-Exchange Chromatography

Honey (1000 g.) containing 21.8 meq. of free acid and 6.2 meq. lactone was diluted to 3.5 l. with distilled water. The solution was then decolorized with 28 g. of deactivated carbon (16) and filtered, and the carbon was rinsed with 100 ml. of distilled water. The combined filtrates were acidified by passing through a column containing 375 ml. Dowex 50 × 8, 50–100 mesh, which was then rinsed with five bed volumes of water.

The honey solution was passed through two 125-ml. Dowex 1 (formate) columns in series. The second column was intended to retain any breakthrough acidity (16). No acids were obtained from this second column. The first Dowex column was rinsed with distilled water until a negative carbohydrate test was obtained (17).

The acids were removed from the column by eluting with a gradient to 4 *N* formic acid (18). The reservoir and mixing chambers contained 590 ml. of 4 *N* formic acid and 1410 ml. of distilled water, respectively. Fractions of 5 ml. were collected (flow rate maintained at 70–100 ml./hr.).

The acid fractions were located by paper chromatography using washed Whatman No. 1 paper and the *n*-butanol–acetic acid–water solvent. Every second tube was evaporated to dryness, 0.5 ml. of distilled water was added to each tube, and aliquots of 0.01 ml. were papergrammed. The acid fractions were combined and concentrated to 5 ml. by evaporation in a filtered air stream at less than 40°C.

The solutions containing several components were resolved by chromatographing on 4 ml. Dowex 1 columns (acetate, −400 mesh). The acids were eluted using a gradient to 4 *N* acetic acid with the flow rate about 1 ml./min.

Fractions of 1.8 ml. were collected and evaporated to dryness, 0.1 ml. of distilled water was added to each residue, and the acids were located by papergramming 10-μl. aliquots.

Where two or more components were not separable by the ion-exchange column, an 8-g. silicic acid column (12) was often capable of separating them. The gradient elution method (18) was used, varying the gradient between pure $CHCl_3$ and approximately 70% *n*-butanol in chloroform, and the acids were located by paper chromatography as described above.

Identification Procedures

Tentative identification of the acids was accomplished by means of paper chromatography using washed chromatographic paper. Solvent systems used for the nonvolatile acids were *n*-BuOH–HOAc–H_2O (4:1:5) (14), *n*-AmOH–5 *N* HCO_2H (1:1) (19), and "solvents" F, B, C, and D of Reio (20). The papers were sprayed with 0.04% bromophenol blue (pH 6.9). Comparison of the R_f values obtained in the six different solvent systems with values recorded in the literature would usually reveal only a small number of possible compounds. These compounds were then papergrammed with the suspected compounds.

The procedures used for the volatile acids were those of Kennedy and Barker (13) and Reid and Lederer (21).

After the tentative identification by paper chromatography, the infrared spectrum of the sodium salt of the unknown acid was compared with those of known salts using the KBr disk technique (Perkin Elmer Model 21 infrared spectrophotometer).

Derivatives (generally the amides and the *p*-nitrobenzyl, *p*-bromophenacyl, and the *p*-phenylphenacyl esters) were prepared in most cases.

In certain cases, the x-ray diffraction patterns of the crystalline sodium salts were of value. All samples were run on a G.E. XRD-3 direct recording x-ray diffraction unit. The samples were placed on glass slides approximately 0.015 in. deep, depending on the amount available.

RESULTS

Silicic Acid Chromatography

The results obtained by titration of the fractions from the large silicic acid column are given in Fig. 1. Figure 1 and Table I give a fairly reliable estimation of the relative quantities of acids present, but are not quantitative.

Fraction A

Acetic and Butyric (Predominating) Acids. Identification was made by paper chromatography. The latter acid was also identified in the fractions from the steam distillation experiment by infrared spectra and the *p*-bromophenacyl ester, m.p. = 60–61°C.; mixed m.p. = 60–61°C.

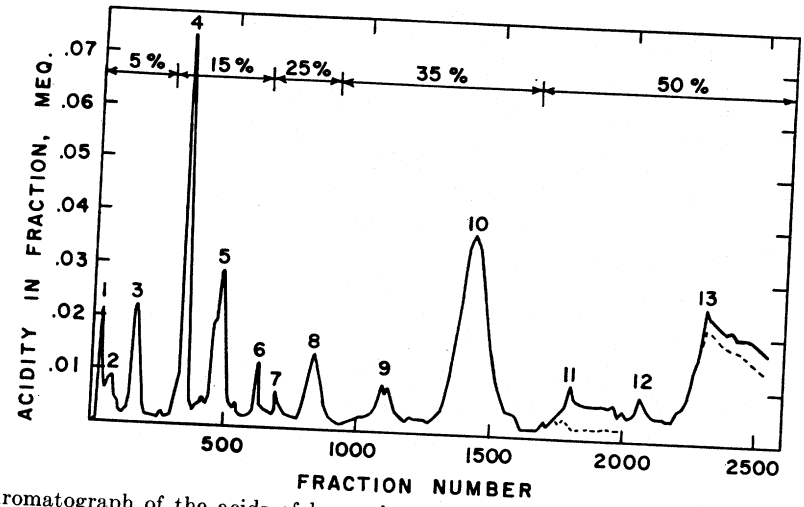

FIG. 1. Chromatograph of the acids of honey by chloroform–butanol elution of a 160-g. silica gel column. The butanol concentration of the eluant is given at the top of the figure. The broken lines for peaks 11 and 13 represent direct titration; the continuous lines for these peaks were obtained by back titration (see text).

Unknown I. A rapidly moving solid acid, m.p. = 152–154°, twice-recrystallized from water, was isolated by silicic acid chromatography from this fraction and the corresponding fraction from the steam distillation. It could not be identified.

TABLE I

FRACTIONATION OF HONEY ACIDS BY SILICIC ACID CHROMATOGRAPHY

Fraction	Fraction No.	Peak No.[a]	Acid content[b]	Identity
			meq.	
A	26–139	1	0.47	Butyric acid
		2	0.37	Unknown I
B	140–195	3	0.70	Acetic acid
C	300–370	4	1.72	Formic acid
D	441–570	5	1.44	Succinic and lactic acids, Unknown II
E	571–760	6	0.29	Pyroglutamic acid
		7	0.24	Unknown III
F	761–940	8	1.02	Possible oxalic, Unknown III
G	986–1200	9	0.90	Malic acid
H	1341–1520	10	5.48	Citric acid
I	1681–2000	11	1.69	Gluconic acid, phosphate esters, inorganic acids
J	2001–2160	12	0.89	
K	2161–2540	13	6.67	

[a] See Fig. 1.

[b] Amount of 41.5 meq. acid applied to column.

Fraction B

Acetic Acid. Identification was made by paper chromatography and infrared spectrum of the sodium salt. Anilide (m.p. 109°C.) and the *p*-nitrobenzyl ester, m.p. and mixed m.p. 74–75°C.

Fraction C

Formic Acid. Identification was made by paper chromatography, the x-ray diffraction pattern, and the infrared spectrum of the crystalline sodium salt. The *p*-bromophenacyl ester was prepared by several procedures, including that of Kubota and Matsuura (22); despite several recrystallizations the melting point was over a range of 94–103°C.

Fraction D

Succinic Acid. The crystalline acid identified by R_f, infrared spectra (sodium salt), the *p*-nitrobenzyl ester (m.p. 81–83°, mixed m.p. 82–85°), and the di-*p*-bromophenacyl ester, m.p. 209.5–211.5°. The x-ray pattern of the latter compound was identical with that of authentic di-*p*-bromophenacyl succinate, m.p. 212.5–213.5°.

The shape of the peak (peak 5, Fig. 1) indicated two components. Paper chromatography revealed three: two acids corre-

sponding to succinic and lactic acids and a third component with R_f of 0.85 in the amyl alcohol solvent. Although succinic and lactic acids have similar rates of movement on the silicic acid column, careful chromatography on smaller silicic acid columns resulted in the separation of these acids.

Lactic Acid. This was identified by R_f, infrared spectra, and the *p*-phenylphenacyl ester (m.p. 135–138°, mixed m.p. 134–137°). The ratio of succinic acid to lactic acid was 1.39 to 1.75 equiv.

Unknown II. R_f = 0.85 (amyl alcohol solvent). Not recovered from second column.

Fraction E

Silicic acid chromatography of this material produced two pure substances:

Pyroglutamic Acid. This was identified by paper chromatography, infrared spectrum, and several reactions. The unknown and authentic pyroglutamic acid were hydrolyzed by heating with 6 *N* HCl (23), and aliquots were papergrammed using *n*-butanol–acetic acid–water (4:1:5) (24). Ninhydrin showed that glutamic acid was produced from both materials. Neither the original unknown nor pyroglutamic acid reacted with ninhydrin. In addition, both known pyroglutamic acid and the unknown gave positive reactions to the Rydon-Smith reagent (25).

Unknown III. Solid material, m.p. 164–166°C. (twice-recrystallized from ethanol); pK > 9.0. It contained no carbon. This material was also found in the residue from the steam distillation.

Fraction F

A small amount of the unidentified component of the previous fraction was found.

Oxalic Acid. The bulk of the acid material was found in an acid fraction having a pK of approximately 3.8. The x-ray diffraction pattern resembled that of sodium oxalate. However, the infrared spectra had failed to confirm oxalic acid, and no derivatives were prepared.

Fraction G

Malic Acid. Identification was made by paper chromatography, infrared spectrum; phenacyl derivative, m.p. and mixed m.p. 107.5–109°C. X-ray powder patterns were identical.

Fraction H

Citric Acid. This was confirmed by paper chromatography, infrared spectrum, and the amide (m.p. 105–108°; mixed m.p. 105–108°).

Fractions I, J and K

Gluconic Acid. These fractions have not been investigated except by paper chromatography. The presence of gluconic acid was later confirmed by infrared spectra of fractions from the residue of steam distillation. The only lactone material recovered was found in these fractions. The presence of lactones was indicated by direct (Fig. 1, dotted line) and back titration (solid line) of Fractions I, J, and K. The difference between the two is presumed to be lactone. The presence of phosphoric acid esters was shown by paper chromatography of the last three fractions.

Ion-Exchange Chromatography

The results found by ion-exchange displacement chromatography are given in Table II. It is probable that any oxalic acid originally present was retained on the column since Palmer (26) found that strenuous conditions were required for its removal.[4]

Fraction I

Unknown IV. This could not be detected after the solution had been concentrated, though low R_f would tend to eliminate volatile acids.

Fraction II

Gluconic Acid. Identified by paper chromatography and the infrared spectrum. Although paper chromatography indicated that the material obtained from the column contained only one acid component, another acid area due to gluconolactone (R_f = 0.41 in the *n*-butanol solvent) generally appeared after the sprayed paper chromatograms were kept at room temperature for several hours.

[4] Since the completion of this work, Joseph H. Schwartz, of this laboratory, has suggested that 0.5 *N* HCl would release oxalic acid retained on the ion-exchange column.

TABLE II

FRACTIONATION OF HONEY ACIDS BY ION-EXCHANGE CHROMATOGRAPHY

Fraction	Tube No.	Normality of eluting formic acid	$R_f \times 100^a$	Identity
		N		
I	36–39	0.005–0.010	2	Unknown IV
II	56–73	0.015–0.040	15	Gluconic acid
III	74–127	0.040–0.36	15; 1; 5; 40 (lactone)	Gluconic acid; Unknowns V and VI (polymers of gluconic acid); gluconolactone
IV	128–134	0.36–0.46	67; 15; 52	Lactic acid; gluconic acid; Unknown VI
V	135–147	0.46–0.54	67; 15; 22; 36	Lactic acid; gluconic acid; Unknowns VII and VIII
VI	148–170	0.54–0.73	49; 16	Pyroglutamic acid; Unknown IX
VII	184–225	0.79–1.15	67; 16	Succinic acid; Unknown X
VIII	226–253	1.15–1.38	43	Malic acid
IX	361–384	2.80–3.06	32	Citric acid

[a] Solvent mixture, butanol–acetic acid–water (4:1:5).

Gluconic acid freshly prepared from calcium gluconate did not display this behavior but did after equilibrating. Paper chromatography of equilibrated unknown solution and gluconic acid prepared from calcium gluconate also revealed acidic areas corresponding to Unknowns V and VI [Unknowns V and VI may be polymers of gluconic acid (27)]. The neutral equivalent obtained after the material had equilibrated was 256.

Fraction III

Ion-exchange chromatography of Fraction III yielded gluconic acid (predominately) and Unknown VI. Unknown V was not recovered.

Unknown VI. Major component, R_f 0.05; traces of gluconic acid and related components. Neutral equivalent, 345. The infrared spectrum could not be matched with the spectrum of any acid investigated thus far, but did indicate a large hydroxyl content.

Gluconic Acid. Identified by paper chromatography, infrared spectrum, and by preparation of gluconamide (28), m.p. 140–141°, decomp. 143–144°, mixed m.p. 140–141°, decomp. 143–144°.

Fraction IV and Fraction V

Fractionated on Dowex 1 (acetate) columns.

Gluconic Acid. Identified by paper chromatography.

Unknowns VI, VII and VIII. Present only in trace amounts and not identified.

Fraction VI

Fractionated on a Dowex 1 (acetate) column.

Pyroglutamic Acid. Identified by paper chromatography and infrared spectrum.

Unknown IX. Not identified.

Fraction VII

Succinic Acid. This was observed by paper chromatography. After fractionation on a 4.0-ml. acetate column, succinic acid was identified; infrared spectrum.

Unknown X. Not identified.

Fractions VIII and IX

Malic and Citric Acids. These were identified by paper chromatography and infrared spectra.

DISCUSSION

The only lactone-forming acid was gluconic acid. Concentration of the fraction that was not retained on the original Duolite column produced a gel, so that the presence of glucuronic acid or polymers cannot be excluded. This fraction was not further studied.

The largest acid fraction in peaks 1–10 from the large-scale experiment (Fig. 1) was the citric acid fraction, containing 5.48 meq.,

or 12 % of the material added to the column. When tubes from peaks 11–13 were titrated, end point fading made the titration difficult. Tubes beyond 1730 (Fractions I, J, and K) were titrated by adding excess alkali and back titrating with acid; this gave values three times higher than direct titration for Fraction I, and about 25 % higher for Fraction K. These fractions, totaling 9.3 meq. (22 % of material put on the Duolite column) contained the gluconic acid held on the Duolite column. About 48 meq. additional material, much of it gluconic acid, passed through the Duolite column; this was 60 % of the acidity applied to the column. In the ion-exchange chromatography, using a stronger exchanger, no acidity passed through. Here Fraction III, gluconic acid and associated polymers, accounted for 9.2 meq. of acidity of the total of 28 applied to the column (33 %), while the citric acid accounted for 1.2 meq. (4.3 %). Gluconic acid was therefore the principal acid of the honey investigated. These results are consistent with those of White *et al.* (11) who found that all of 225 samples of honey contained lactone material, having an average of 21.5 meq./kg. of free acid and 8.92 meq./kg. of lactone.

ACKNOWLEDGMENTS

We acknowledge with gratitude the assistance of Carl T. Leander in determining the infrared spectra, of C. R. Eddy in their interpretation, and of David A. Lutz in obtaining the x-ray diffraction data.

REFERENCES

1. Farnsteiner, K., *Z. Nahr. Genussm.* **15,** 598 (1908); *Chem. Zentr.* **79 II,** 189 (1908).
2. Fincke, H., *Z. Nahr. Genussm.* **23,** 255 (1912); *Chem. Zentr.* **83 I,** 1584 (1912).
3. Heiduschka, A., *Pharm. Zentralhalle* **52,** 1051 (1911); *Chem. Zentr.* **82 II,** 1545 (1911).
4. Nelson, E. K., and Mottern, H. H., *Ind. Eng. Chem.* **23,** 335 (1931).
5. Philipsborn, H. von, *Protoplasma* **41,** 415 (1952).
6. Vavruch, I., *Chem. Listy* **46(2),** 116 (1952).
7. Angeletti, A., *Giorn. farm. chim.* **81,** 533 (1932); *C. A.* **27,** 1957 (1933).
8. Goldschmidt, S., and Burkert, H., *Z. physiol. Chem.* **301,** 78 (1955).
9. Cocker, L., *J. Sci. Food Agr.* **2,** 411 (1951).
10. Walton, G. P., *J. Assoc. Offic. Agr. Chemists* **27,** 477 (1944).
11. White, J. W., Jr., Petty, J., and Hager, R. B., *J. Assoc. Offic. Agr. Chemists* **41,** 194 (1958).
12. Bulen, W. A., Varner, J. E., and Burrell, R. C., *Anal. Chem.* **24,** 187 (1952).
13. Kennedy, E. P., and Barker, H. A., *Anal. Chem.* **23,** 1033 (1951).
14. Buch, M. L., Dryden, E. C., and Hills, C. H., *J. Agr. Food Chem.* **3,** 960 (1955).
15. Marvel, C. S., and Rands, R. D., Jr., *J. Am. Chem. Soc.* **72,** 2642 (1950).
16. Richardson, A., and Hulme, A. C., *J. Sci. Food Agr.* **8,** 326 (1957).
17. Feigl, F., "Spot Tests in Organic Analysis," 5th Engl. ed., p. 390. Elsevier Publishing Company, Amsterdam, 1956.
18. Paar, C. W., Proc. Biochem. Soc. 324th Meeting, XXVII, *in* Bock, R. M., and Ling, N.-S., *Anal. Chem.* **26,** 1543 (1954).
19. Buch, M. L., Montgomery, R., and Porter, W. L., *Anal. Chem.* **24,** 489 (1952).
20. Reio, L., *J. Chromatog.* **1,** 338 (1958).
21. Reid, R. L., and Lederer, M., *Biochem. J.* **50,** 60 (1951).
22. Kubota, T., and Matsuura, T., *J. Inst. Polytech. Osaka City Univ.* **1,** 49 (1950); *C. A.* **46,** 2011 (1952).
23. Bethke, R. M., and Steenbock, H., *J. Biol. Chem.* **58,** 105 (1923).
24. Smith, I., "Chromatographic Techniques," p. 67. Interscience Publ., New York, 1958.
25. Rydon, H. N., and Smith, P. W. G., *Nature* **169,** 922 (1952).
26. Palmer, J. K., *Conn. Agr. Expt. Sta. Bull. No.* **589** (1955).
27. Green, J. W., *in* "The Carbohydrates: Chemistry, Biochemistry, Physiology" (Pigman, W.), p. 305. Academic Press, New York, 1957.
8. Glattfield, J. W. E., and Macmillon, D., *J. Am. Chem. Soc.* **56,** 2481 (1934).