with it. Still, the trend of absorption frequency for the complexes in such a series generally appears to follow the trend of the frequencies of the first absorptions of the substituted benzenes. However, the complexes of a given halobenzene with different halogens do not obey the rule. It has already been suggested[2b] on other grounds that there may be complexing by way of the halogen substituent in these cases as well as or instead of by the mechanism effective in hydrocarbons.

The crudity of the numerical treatment we have had to employ should not obscure the very striking qualitative evidence that the absorption of the complex is determined principally by the aromatic part. This experimental finding is most reasonably interpreted by ascribing the transition either to a shifted aromatic band, as we have done here, or to an electron transfer between aromatic and halogen. The latter alternative was deemed unlikely by Mulliken on the basis of the intensities. The shifted aromatic band remains as the most likely explanation; the appearance of such a band requires that the symmetry of the free molecule be disturbed. In this case, then, the benzene–halogen complexes could not have the structure favored by Keefer and Andrews,[2b] with the halogen molecule aligned along the hexagonal axis.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA]

Chemical Methods for Estimating the Distribution of C^{14} in Biosynthetic Glucuronic Acid[1]

BY FRANK EISENBERG, JR.,[2] AND SAMUEL GURIN

In order to determine the location and specific activity of C^{14} in biosynthetic glucuronic acid derived from radioactive precursors, several chemical degradations have been developed which yield a series of fragments sufficient to permit the measurement or calculation of C^{14} in all of the carbon atoms of glucuronic acid. The pyranose structure assigned to conjugated glucuronides has been confirmed.

Although the phenomenon of detoxification by glucuronic acid has engaged the attention of biochemists for nearly a century, the origin of this compound in the animal organism has remained obscure. Numerous hypotheses have been suggested to account for its origin, but none has been definitive.[3-8] With the advent of tracer techniques it has become clear that it is now possible to investigate the biosynthesis of glucuronic acid from precursors labeled with C^{14}.

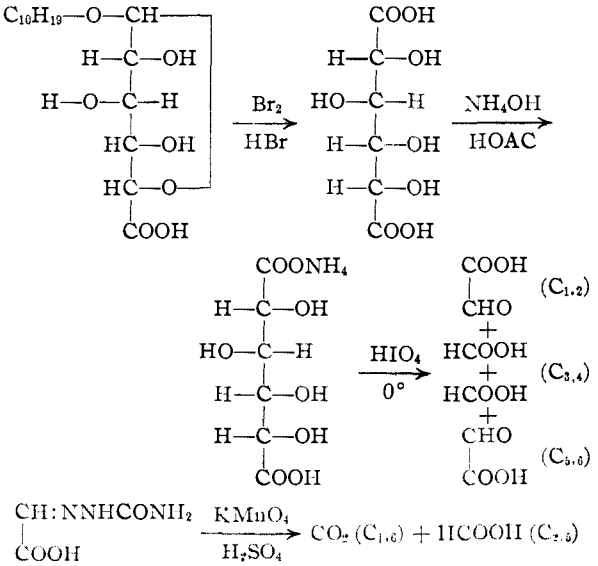


Fig. 1.

As a preliminary to this study, it was necessary to develop chemical degradations designed to locate the position and amount of C^{14} in the individual carbon atoms of radioactive glucuronic acid. The present paper describes several chemical degradations which yield five fragments: $C_{1,6}$, $C_{2,5}$, $C_{3,4}$, $C_{1,2}$ and $C_{4,5,6}$. Recent tracer studies on the biosynthesis of glucuronic acid[9] have utilized only the classical decarboxylation reaction, yielding C_6. From the C^{14} content of the above-mentioned fragments including C_6, it is possible to calculate the specific activity of all six carbon atoms of glucuronic acid.

Procedure 1 (See Fig. 1).—Menthyl glucuronide, isolated from rabbit urine, was oxidatively hydrolyzed to saccharic acid which was subsequently cleaved by periodic acid to formic acid and glyoxylic acid. Sprinson and Chargaff[10] reported that tartaric acid can be oxidized at low temperature with the utilization of 1 mole of HIO_4. Since no CO_2 was evolved under these conditions, the tartaric acid was presumably cleaved to 2 molecules of glyoxylic acid. An analogous reaction has been found with saccharic acid. By measuring the utilization of HIO_4 at 0°, it was observed that exactly 3 moles of HIO_4

(1) Aided by a grant from the American Cancer Society administered by the Committee on Growth of the National Research Council. The authors wish to thank the Analytical Laboratories of Sharp and Dohme, Inc., and the Eastern Regional Research Laboratory for some of the analyses.

(2) Presented by Frank Eisenberg, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Pennsylvania. Predoctoral fellow of the National Institutes of Health, United States Public Health Service, 1949-1950.

(3) O. Schmiedeberg and H. Meyer, *Z. physiol. Chem.*, **3**, 422 (1879).
(4) E. Fischer and O. Piloty, *Ber.*, **24**, 521 (1891).
(5) H. Thierfelder, *Z. physiol. Chem.*, **10**, 163 (1886).
(6) F. Schmid, *Compt. rend. soc. biol.*, **123**, 223 (1936).
(7) A. J. Quick, *J. Biol. Chem.*, **70**, 59, 397 (1926).
(8) W. L. Lipschitz and E. Bueding, *ibid.*, **129**, 333 (1939).
(9) E. H. Mosbach and C. G. King, *ibid.*, **185**, 491 (1950).
(10) D. B. Sprinson and E. Chargaff, *ibid.*, **164**, 433 (1946).

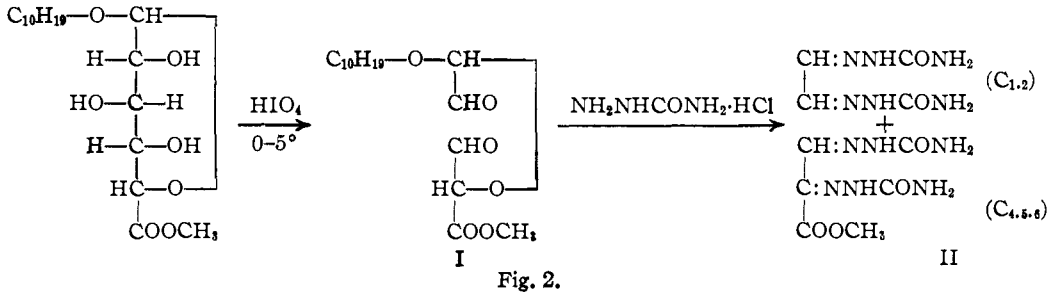


Fig. 2.

was consumed; at the same time no CO_2 was evolved. It was inferred that the products of the reaction were glyoxylic acid and formic acid. Both of these compounds were isolated from the reaction mixture and characterized unequivocally. Formic acid is derived from $C_{3,4}$, the aldehyde moiety of glyoxylic acid from $C_{2,5}$, while the carboxyl group of glyoxylic acid is derived from $C_{1,6}$ of the original glucuronic acid (Fig. 1). The two carbon atoms of glyoxylic were subsequently separated by conversion to the semicarbazone followed by oxidation with $KMnO_4$.[11] This procedure yields 3 fragments $C_{1,6}$, $C_{2,5}$ and $C_{3,4}$.

Procedure 2 (See Fig. 2).—On the basis of previous studies of the structure of bornyl glucuronide,[12] it was assumed that menthyl glucuronide similarly exists predominantly in the pyranose form. Although direct oxidation of menthyl glucuronide with HIO_4 was studied, it was not until the carboxyl group was methylated that the compound yielded an identifiable fragment. The main product of this oxidation was L′-(L-menthoxy)-D-carbomethoxy-diglycolic aldehyde (I) a compound analogous to the dialdehyde similarly obtained from methyl glucoside.[13] The other product was formic acid. Since this reaction was not quantitative, formic acid could have arisen from other carbons than C_3; hence, C_3 is not available for isotopic analysis by this procedure.

L′-(L-Menthoxy)-D-carbomethoxy-diglycolic aldehyde was invariably decomposed in the presence of carbonyl reagents to give derivatives of degradation products of the parent molecule. It was thus impossible to prepare a derivative of the intact compound. Tentative identification was made by elementary and functional group analyses and a molecular weight determination. Attempts to convert the compound to the corresponding acid or alcohol failed. Acid hydrolysis yielded menthol and glyoxal, but nothing representing the other aldehyde moiety could be recovered from the hydrolysate. In the presence of semicarbazide hydrochloride, however, hydrolysis occurred rapidly with the simultaneous condensation of the reagent with the aldehyde groups, producing menthol, glyoxal disemicarbazone, and the disemicarbazone of mesoxaldehyde methyl ester (II), a compound which apparently has not been described. Characterization of these 3 compounds served to establish the structure of the parent dialdehyde and justified the assumption of the pyranose structure for menthyl glucuronide.

The formation of mesoxaldehyde methyl ester disemicarbazone was entirely unexpected since on the basis of the structure of the dialdehyde it would be anticipated that either the semicarbazone of tartronic acid semialdehyde or the semicarbazone of the isomeric hydroxypyruvic acid should have been formed. That a disemicarbazone was obtained indicated that the hydroxyl group of the three-carbon moiety was oxidized during the formation of the compound, a reaction that is reminiscent of osazone formation with phenylhydrazine. To our knowledge such behavior on the part of semicarbazide is unprecedented. Clearly the oxidation could not have occurred during the earlier formation of the parent dialdehyde since the analytical data for that compound are consistent with the structure shown in Fig. 2.

Our results are in accord with those of Huebner, *et al.*,[14] who studied the oxidation of bornyl glucuronide by HIO_4. From 1 mole of bornyl glucuronide they obtained a mole of oxalic acid, a mole of bornyl formate, and three equivalents of acid, presumably formic acid. To account for the oxalic acid resulting from this oxidation it was necessary to postulate the addition of an atom of oxygen at the carbon atom corresponding to the original C_5. Subsequent hydrolysis of the acetal and cleavage of the three-carbon moiety would yield oxalic acid. These authors further postulated the formation prior to this oxidation of the bornyl analog of L′-(L-menthoxy)-D-carbomethoxy-diglycolic aldehyde. Thus we have succeeded in confirming their postulates, not only by the isolation of the dialdehyde, but by observing also the oxidation of the potential hydroxyl group of the three-carbon moiety of the dialdehyde by means of another "oxidant" (semicarbazide hydrochloride). Procedure 2 thus yields two fragments, $C_{1,2}$ and $C_{4,5,6}$.

These chemical degradations are being applied to specimens of radioactive menthyl glucuronide obtained following the administration of various precursors labeled with C^{14}. Including the decarboxylation, they can be carried out with as little as 0.7 g. of crude ammonium menthyl glucuronidate.

Experimental

Preparation of Menthyl Glucuronide.—Rabbits weighing about 2 kg. were fed *ad libitum* a diet consisting of a standard rabbit chow containing 2% *l*-menthol. The drug was incorporated in the chow in an ether solution followed by air-drying to remove the solvent. The animals consumed an average of 2–3 g. of menthol a day. Glucuronic acid was isolated from the urine as ammonium methyl glucuronidate

(11) I. M. Buchanan, J. C. Sonne and A. M. Delluva, *J. Biol. Chem.*, **173**, 81 (1948).

(12) J. Pryde and R. T. Williams, *Biochem. J.*, **27**, 1197 (1933).

(13) E. L. Jackson and C. S. Hudson, THIS JOURNAL, **59**, 994 (1937).

(14) C. F. Huebner, R. Lohmar, R. J. Dimler, S. Moore and K. P. Link, *J. Biol. Chem.*, **159**, 503 (1945).

by the method of Williams.[15] The crude ammonium salt was freed of gross organic impurities by recrystallization in 5% solution from 50% $(NH_4)_2SO_4$. A few drops of concentrated ammonia were added to prevent hydrolysis of the acetal linkage. Recovery was quantitative, yielding a white crystalline product. The ammonium salt was dissolved in enough $N/2$ H_2SO_4 to make a 5% solution, and the liberated menthyl glucuronide extracted with six equal volumes of ether. The ether phase was evaporated by aeration to a thick sirup which was then dissolved in warm water to make a 10% solution, filtered, and chilled for several hours. The resulting white crystalline precipitate was removed by filtration, washed twice with cold water, and dried *in vacuo* over $CaCl_2$. Recovery was 70%. The aniline adduct, $C_{38}H_{63}O_{14}N$, was prepared for characterization[16]; m.p. 181°, literature 182°.

Preparation of Silver Menthyl Glucuronidate.—Although menthyl glucuronide is a crystalline compound, its melting point is not sufficiently reproducible to serve as a criterion of purity. Organic derivatives, while adequate for characterization, are unsuitable as purification derivatives for isotopic compounds because of the large carbon dilutions incurred. None of the metal salts reported in the literature had either melting or decomposition points. The silver salt, however, was found to have a reproducible decomposition point. To prepare the silver salt, menthyl glucuronide dissolved in cold absolute EtOH in 10% solution was treated with saturated alcoholic $AgNO_3$ followed by alcoholic ammonia to *p*H 5, whereupon a white flocculent precipitate appeared. The suspension was chilled, centrifuged in the cold, and the precipitate washed three times with cold absolute EtOH. During this time the precipitate became gelatinous. It was then washed twice with cold ether, air-dried in the dark, then further dried *in vacuo* over P_2O_5 to a white sandy powder; m.p. 165° (dec.); yield 72%. Calcd. for $C_{16}H_{28}O_7Ag$: Ag, 24.6. Found: Ag, 24.6.

Conversion of the Silver Salt to Menthyl Glucuronide.—The silver salt was not stable and darkened within 24 hours. It was, therefore, immediately converted to the ammonium salt by treating an aqueous suspension with a slight excess of NH_4Cl, filtering off the AgCl, and adding $(NH_4)_2SO_4$ to make a 50% solution. The resulting white crystalline product was removed by filtration and dried *in vacuo* over $CaCl_2$. Menthyl glucuronide was prepared from the ammonium salt by the procedure described above. Since this product was obtained from the pure silver salt by way of the ammonium salt, it was necessary only to evaporate the ether to recover pure menthyl glucuronide from the extract. The yield based on the silver salt was quantitative.

Preparation of Ammonium Acid Saccharate.—Menthyl glucuronide was oxidatively hydrolyzed with Br_2 and HBr by the procedure of Neuberg and Neimann.[17] At the end of the oxidation period the mixture was extracted three times with ether to remove brominated menthol derivatives and excess Br_2. The solution was made ammoniacal and evaporated to a small volume at 100° in a stream of nitrogen, then chilled and acidified with glacial acetic acid, yielding an immediate white crystalline precipitate. Chilling was continued for several hours after which the precipitate was removed by centrifugation and washed once with cold absolute alcohol. It was then dissolved in a small volume of hot water, the solution treated with norit and filtered. The compound was precipitated in crystalline form by the addition of five volumes of alcohol. After chilling several hours the compound was removed by filtration, washed twice with cold absolute alcohol, and dried *in vacuo* over $CaCl_2$. *Anal.* Calcd. for $C_6H_{13}O_8N$: N, 6.17; equiv. wt., 227. Found: N, 6.26; equiv. wt., 227. $[\alpha]_D$ +5.84° lit.[18] Found: $[\alpha]_D$ +5.47° (2% in water). Hence the compound is ammonium acid saccharate; yield 60%.

Cleavage of Ammonium Acid Saccharate to Glyoxylic and Formic Acids.—A 1% solution of ammonium acid saccharate was treated with 4 molar equivalents of HIO_4 at 0° for 1 hour. The reaction was stopped by adding sufficient HI (as KI + H_2SO_4) to destroy the HIO_3 and excess HIO_4. The resulting precipitated iodine was removed by centrifugation and washed three times with cold water, combining supernatant and washes. The iodine remaining in solution was removed by treatment with N thiosulfate, added dropwise to avoid a large excess. An excess of 200–300% of semicarbazide hydrochloride was added and the solution allowed to stand 30 minutes at room temperature. It was then neutralized to *p*H 7 with solid $NaHCO_3$ and evaporated to a small volume *in vacuo* at 55° (bath temperature), then acidified to *p*H 1 with 6 N H_2SO_4 and chilled several hours, yielding a heavy white crystalline precipitate. The precipitate was washed twice by centrifugation with a small volume of cold water, recrystallized from hot water, then washed and dried *in vacuo* over $CaCl_2$; m.p. 200°, mixed with known glyoxylic acid semicarbazone, 200°; neut. equiv. calcd. 131, found 132. The compound is, therefore, glyoxylic acid semicarbazone; yield 50–60%. The aldehyde carbon represents carbons 2 and 5; and the carboxyl carbons 1 and 6 of the original glucuronic acid.

The liquid from which the semicarbazone was centrifuged was steam distilled and the distillate was treated with $N/10$ I_2, added dropwise, to remove SO_3 generated during the distillation (from the decomposition of excess thiosulfate). The distillate was then filtered to remove precipitated sulfur and was divided in half.

One-half was neutralized with NaOH and evaporated to dryness. A *p*-bromophenacyl ester was prepared from the sodium salt remaining after evaporation; m.p. 134–136°, mixed with known *p*-bromophenacyl formate, 135°. This step is not a part of the degradation, but serves only to identify the formic acid.

The other half of the distillate was boiled with a solution of $HgSO_4$ in dilute H_2SO_4 while sweeping with nitrogen into saturated $Ba(OH)_2$. Carbon dioxide was evolved, a reaction that is virtually specific for formic acid. The compound is, therefore, formic acid and represents carbons 3 and 4 of the original glucuronic acid. The yield of $BaCO_3$ from formic acid, based on the weight of saccharate used, was 55–65%.

Degradation of Glyoxylic Acid Semicarbazone.—For the separation of the two carbon atoms of glyoxylic acid the semicarbazone was oxidized with permanganate according to the procedure of Buchanan, *et al.*[11] By this method two separate portions of CO_2 are produced; one being derived from the carboxyl and semicarbazide carbons and representing carbons 1 and 6 of the original glucuronic acid (yield 40%); the other is derived from the aldehyde group and represents carbons 2 and 5 (yield 50–60%).

Cleavage of Methyl Menthyl Glucuronidate to L′-(L-Menthoxy)-D-carbomethoxy-diglycolic Aldehyde.—A 10% solution of menthyl glucuronide in ether was treated with an ethereal solution of diazomethane. The ether was evaporated, leaving a quantitative yield of the amorphous, hygroscopic methyl menthyl glucuronidate, a compound that apparently has not been described. Repeated attempts to crystallize this compound failed. A 2% solution of the methyl ester in a 75/25 dioxane–water solution was treated with 4 moles of HIO_4 per mole of ester at 0–5° for eight days. The dioxane was purified by a procedure described elsewhere.[19] Toward the end of the oxidation period, fine white needles precipitated which were identified as iodic acid. Nothing was done to remove them at this point. At the end of the reaction period an equal volume of cold dioxane was added, followed by the dropwise addition of saturated aqueous KCl, which precipitated the potassium salts of iodic and periodic acids quantitatively. The suspension was allowed to stand in the cold for several hours to complete the precipitation of the salts which were then removed by filtration and washed with cold dioxane. The combined washes and filtrate were shaken with solid $NaHCO_3$ in the cold until the newly-formed HCl was neutralized. NaCl and unused $NaHCO_3$ were removed by filtration and washed with cold dioxane. The combined washes and filtrate were evaporated to a small volume *in vacuo* at 50° bath temperature until a yellow oil separated. The suspension was cooled until the globules solidified, and was then filtered. The solid was dissolved in ethanol in 10% solution and treated with norit for one-half hour. The solution was filtered and diluted with five volumes of water added slowly with stirring to avoid local precipitation of the oil. Upon chilling, the turbid emulsion crystallized as fine white needles. The precipitate was removed by filtration, washed with cold water, and dried *in vacuo* over $CaCl_2$; sintering point 120°, m.p. 134–135°; yield 50%. *Anal.* Calcd. for

(15) R. T. Williams, *Biochem. J.*, **34**, 272 (1940).

(16) M. Bergmann and W. W. Wolff, *Ber.*, **56**, 1060 (1923).

(17) C. Neuberg and W. Neimann, *Z. physiol. Chem.*, **44**, 127 (1905).

(18) O. Sohst and B. Tollens, *Ann.*, **245**, 1 (1888).

(19) E. J. Eigenberger, *J. prakt. Chem.*, **130**, 75 (1931).

$C_{16}H_{26}O_8$: C, 61.2; H, 8.28; CH_3O, 9.88; mol. wt., 314. Found: C, 61.2; H, 8.44; CH_3O, 10.0; mol. wt., 304.[20]

Cleavage of L'-(L-Menthoxy)-D-carbomethoxy-diglycolic Aldehyde.—A 1% solution of the dialdehyde in 90/10 methanol–water was heated under reflux at 70° for one-half hour in the presence of twice the quantity of semicarbazide hydrochloride needed to react with four carbonyl groups (two already present, two formed on hydrolysis). During the heating a heavy white precipitate appeared and was removed at the end of the reaction period by centrifugation, and washed twice with hot 90% methanol. Since this compound was insoluble in all organic solvents and water it could not be recrystallized. It was, however, found to be soluble in cold 3 *N* NaOH. After filtering the alkaline solution, the compound was precipitated by acidification with cold 6 *N* H_2SO_4. It was then washed neutral and dried *in vacuo* over $CaCl_2$; m.p. 273°, m.p. mixed with known glyoxal disemicarbazone, 273°. During the early attempts to prepare derivatives of the parent dialdehyde numerous derivatives of glyoxal resulted, all melting at the correct temperatures, alone and mixed with authentic derivatives of glyoxal. Hence the two-carbon moiety is glyoxal and represents carbon 1 and 2 of the original glucuronic acid; yield 75–90%.

(20) Cryoscopic in Exaltone.

The supernatant liquid left after the removal of the glyoxal derivative was evaporated *in vacuo* to a small volume and extracted with ether. The ether extract was evaporated to dryness leaving a white crystalline solid having the appearance and odor of menthol. This residue was treated with 3,5-dinitrobenzoyl chloride, yielding a crystalline derivative melting at 153°; m.p. mixed with known menthyl 3,5-dinitrobenzoate, 152°. Hence the dialdehyde contains the menthyl group. This step is not a part of the dehydration, but serves only to identify the menthol moiety.

The aqueous solution left after ether extraction was chilled several hours, yielding a cream-colored precipitate, which was readily recrystallized from 50% methanol, washed twice with cold water, and dried *in vacuo* over $CaCl_2$; m.p. 215°. *Anal.* Calcd. for $C_6H_{10}O_4N_6$: C, 31.3; H, 4.35; N, 36.5; CH_3O, 13.5. Found: C, 31.31; H, 4.42; N, 36.47; CH_3O, 13.47. Hence the compound is the disemicarbazone of mesoxalaldehyde methyl ester and represents carbons 4, 5 and 6 of the original glucuronic acid; yield 40–50%.

Determination of Periodic Acid.—HIO_4 was determined iodometrically by the procedure described by Jackson.[21]

(21) E. L. Jackson, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, 1944, p. 361.

RECEIVED APRIL 6, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF NEW HAMPSHIRE]

The Nitration of Melamine and of Triacetylmelamine[1]

BY EDWARD R. ATKINSON

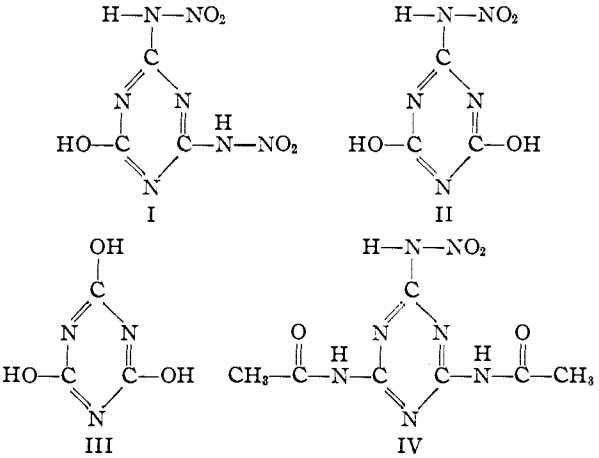
The substance $C_3H_3O_5N_7$ which Cason[3] obtained by the nitration of melamine in acetic anhydride has been identified as N,N'-dinitroammeline (I). The substance "TM-1" ($C_9H_9O_{12}N_{15}$) which Cason obtained by the nitration of triacetylmelamine in acetic anhydride has been identified as nitroammelide ($C_3H_3O_4N_5$) (II) and its hydrolysis product "TM-2" ($C_9H_9O_9N_9$) as cyanuric acid ($C_3H_3O_3N_3$) (III). Fuming nitric acid alone at 25° converted triacetylmelamine to N-nitro-N',N''-diacetylmelamine (IV).

While examining synthetic routes to *sym*-trinitromelamine we have had occasion to repeat earlier work of Whitmore[2] and of Cason,[3] who studied the direct nitration of melamine, and of Cason[3] who studied the nitration of *sym*-triacetylmelamine.

By the nitration of melamine with nitric acid in acetic anhydride at 5° we have obtained the explosive substance $C_3H_3O_5N_7$ for which Cason offered no structural formula. We have observed that the substance can be titrated as a dibasic acid and that it is hydrolyzed quantitatively to cyanuric acid. It is clear that the substance must be N,N'-dinitroammeline (I).

By the nitration of *sym*-triacetylmelamine with nitric acid in acetic anhydride at 20–25° Cason obtained a substance designated TM-1 to which he assigned the formula $C_9H_9O_{12}N_{15}$. This was observed to hydrolyze with the loss of three molecules of nitrous oxide to form a very stable substance, TM-2, $C_9H_9O_9N_9$. In repeating this work we have noted that the substance TM-2 is cyanuric acid, $C_3H_3O_3N_3$ (III). It is apparent that the substance TM-1 is nitroammelide, $C_3H_3O_4N_5$ (II). On the basis of this formula one mole of nitrous oxide is liberated during hydrolysis.

We have made the additional observation that fuming nitric acid alone at 20–25° converts triacetylmelamine to N-nitro-N',N''-diacetylmelamine (IV).



Because of the large volumes of nitrating agent required in these reactions it is apparent that there is ample water (or its solvolytic equivalent) to account for the observed loss of exocyclic nitrogen atoms and acetyl groups. The hydrolysis of the nitration products with loss of nitrous oxide is typical of nitramines.[4]

Experimental Part

Nitration of Melamine.—Commercial Monsanto melamine was purified by a procedure similar to that of Salley

(1) This work is based on work done for the Department of the Army under Contract DA19-020-ORD-12 with Arthur D. Little, Inc.

(2) Work carried out at Pennsylvania State College under the direction of F. C. Whitmore under a contract with the Office of Scientific Research and Development (OSRD Report 351).

(3) James Cason, THIS JOURNAL, **69**, 495 (1947).

(4) Lambertson, Lindley and Speakman, *J. Chem. Soc.*, 1650 (1949).