General Conclusions

1. The presence of an organic base markedly increases the extent of the reduction of nitrobenzene to azoxybenzene by sodium methylate.

2. The presence of an organic base causes some further reduction of azoxybenzene to azobenzene.

3. Increasing molar concentrations of organic bases present in the reaction mixtures caused increased percentage reductions of nitrobenzene and of azoxybenzene.

4. Formaldehyde, one of the predicted intermediate products of the reaction mechanism scheme, is formed in the reaction mixtures containing iso-amylamine, with which it combines to form iso-amylmethylene-imide.

5. The formaldehyde so combined with the iso-amylamine can be isolated and quantitatively determined according to a newly proposed method.

6. The yield of formaldehyde conforms to the equation for a new reaction, $2C_6H_5NO_2 + 3CH_3ONa \longrightarrow (C_6H_5N)_2O + 3CH_2O + 3NaOH$, which is concurrent with Klinger's reaction, $4C_6H_5NO_2 + 3CH_3ONa \longrightarrow 2(C_6H_5N)_2O + 3HCOONa + 3H_2O$.

7. The distribution of the extent of the reduction of nitrobenzene in the presence of iso-amylamine has been calculated and found to conform to the equation for the concurrent reactions noted in (6).

8. The observations of this study afford additional evidence for the previously proposed reaction mechanism schemes involving the apparent acidic dissociation of sodium hydroxide.

CINCINNATI, OHIO

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

THE ACTION OF DIAZOMETHANE ON THE PYRIMIDINE CONSTITUENTS OF NUCLEIC ACIDS

BY FRANCIS H. CASE WITH ARTHUR J. HILL

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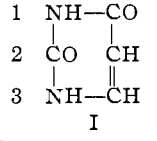
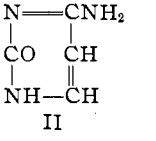
Notwithstanding the large amount of work which has been devoted to the study of nucleic acids, the position of attachment of the carbohydrate nucleus to the pyrimidine constituents (uracil, I, cytosine, II and thymine, III) still awaits definite solution, although the burden of evidence[1] appears to be increasingly in favor of linkage in position 3 rather than 4.



I



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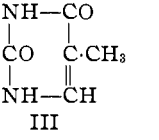
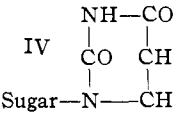


III

[1] Burian, *Ber.*, **37**, 708 (1904); Johnson and Clapp, *J. Biol. Chem.*, **5**, 163 (1908); Wheeler and Johnson, *ibid.*, **3**, 183 (1907); *cf.* Ref. 2.

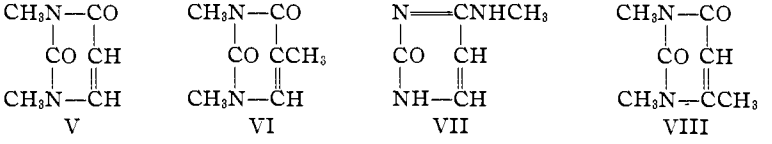
Color tests, as well as reactions, have pointed to this conclusion. For example, the recent work of Levene[2] and his co-workers on the behavior of uracil and its nucleoside uridine, IV, toward either hydrazine or phenylhydrazine (after pretreatment with bromine) is extremely significant in their bearing on this point.

IV



The chief desideratum in solving this interesting problem is a reagent, or reaction, which will effectively introduce a substituent into position 3 (or 4), provided, of course, the position is not already occupied by the sugar residue. An attempt to this end was made by Levene[3] in his alkylation experiments on the silver, lead and alkali salts of the nucleosides, cytidine and uridine. Unfortunately the results were inconclusive.

Diazomethane is not infrequently a useful reagent for methylation, as it may be used in anhydrous neutral media and at room temperatures. It would seem that by appropriate choice of conditions, it might be possible to use this reagent for the methylation either of nucleic acids or their partial breakdown products, the nucleotides, or nucleosides. Subsequent examination of the *methylated pyrimidines*, after hydrolysis of the methylated complex, should throw some light on the position of the sugar linkage. Thus if 1,3-dimethyl-uracil, V, were obtained, one would be justified in



V VI VII VIII

assuming linkage in position 4. On the other hand, the isolation of 1-methyluracil would be evidence of a sugar linkage at position 3.

Although it is our ultimate purpose to investigate the methylation of the nucleic acids, or the nucleosides, it has been necessary first to examine the behavior of the pyrimidine constituents alone toward diazomethane. This paper, then, deals largely with this phase of the problem although some preliminary data on the methylation of yeast nucleic acid are also given.

Uracil, thymine and cytosine have been treated with diazomethane under various conditions, which are described below. In the cases of uracil and thymine, the methylation proceeded smoothly and there was no evidence of the formation of methylated products, other than 1,3-dimethyl-uracil, V, and 1,3-dimethylthymine, VI, respectively. The action of diazomethane on 4-methyluracil was also investigated in order to determine whether or not substitution in position 4 would retard methylation in position 3. However, this compound methylated quite as readily as uracil

[2] Levene, *J. Biol. Chem.*, **63**, 653 (1925); Levene and Bass, *ibid.*, **71**, 167 (1926); Levene, Bass and Simms, *ibid.*, **70**, 229 (1926).

[3] Levene and La Forge, *Ber.*, **45**, 608 (1912).

itself, giving 1,3,4-trimethyluracil, VIII. With cytosine, methylation was very hard to achieve; in fact the slowness and incompleteness of the reaction, and the difficulty in removing polymerized diazomethane from the ether-soluble material, made the investigation of the methylation products very troublesome. However, after considerable experimentation a rather small amount of monomethylcytosine, VII,[4] was isolated. A very interesting observation was made in regard to the ether-insoluble, non-methylated residue. On crystallization from aqueous acetone, this material separated in the form of short prismatic aggregates, quite different from the usual platy, or occasionally spear-shaped, crystals of cytosine. This prismatic variety possessed, however, the same melting point and degree of hydration as ordinary cytosine, and yielded the same picrate. After *dehydration* and crystallization from water or aqueous acetone, conversion to the ordinary cytosine took place. The prismatic variety could not, on the other hand, be prepared by crystallization of ordinary cytosine. So far as the writers are aware, this action of diazomethane is quite unique. The existence of two crystalline forms of cytosine is interesting in view of the fact that phenyl isocytosine has been shown to occur in four different forms.[5]

Preliminary experiments on the methylation of yeast nucleic acid did not lead to any definite evidence concerning the position of attachment of sugar to the pyrimidines. Although the nucleic acid apparently combined with a relatively large amount of diazomethane, subsequent hydrolysis of the methylated material yielded neither methylated nor unmethylated pyrimidines, when the usual procedure for isolation was used. Seemingly the long contact with a large excess of diazomethane had produced profound side reactions.[6] A change in the conditions of methylation will be studied.

Experimental Part

Diazomethane.—The diazomethane used in this investigation was prepared from nitrosomethylurethan by von Pechmann's method.[7] The concentration of the ethereal solutions of diazomethane was determined by Marshall and Acree's method,[8] which involves treatment of the unknown solution with an excess of *m*-nitrobenzoic acid, followed by titration of the unmethylated acid with standard alkali.

Methylation of Uracil. 1,3-Dimethyluracil, V.—In all experiments on the methylation of uracil, 1-g. samples of this substance were treated with ethereal diazomethane solutions of appropriate strengths in 250-cc. flasks provided with air condensers. The progress of reaction in the different flasks was followed from day to day, with particular regard to the disappearance of the yellow color of the reagent and to the solution of the uracil. Some typical data are given in Table I.

[4] *Cf.* Case and Hill, THIS JOURNAL, **51,** 1590 (1929).

[5] Johnson and Hill, *ibid.*, **36,** 1201 (1914).

[6] *Cf.* Levene, *J. Biol. Chem.*, **55,** 437 (1923).

[7] Von Pechmann, *Ber.*, **28,** 855 (1895).

[8] Marshall and Acree, *ibid.*, **43,** 2323 (1910).

TABLE I

THE ACTION OF DIAZOMETHANE ON URACIL

Diazomethane[a] (mol. proportions)	1	3	4	6	1[d]
Time, days	6	5	6[c]	6[c]	1
Ether-sol. material,[b] g.	0.25	0.80	1.22	1.11	0.30

[a] In ether solution. [b] Calculated for complete dimethylation, 1.25 g. [c] Complete solution was effected and the color of the diazomethane had nearly disappeared. [d] With an equal volume of alcohol. Complete decolorization with only slight solution of uracil.

On completion of the designated period of methylation, the various ethereal solutions were filtered, and the filtrates evaporated to dryness. The last line in Table I gives the weight of the residues thus obtained. The figures are only close approximations of the amount of methylated material as the residues were contaminated to some extent with polymerized diazomethane.

The insoluble material, resulting from experiments in which methylation was not complete, melted in no case under 300°. The three possible nitrogen-methylated uracils have the following melting points, respectively: 1-methyluracil, 174–175°; 3-methyluracil, 223–226°; and 1,3-dimethyluracil, 121–122°. Uracil melts at 338°. There was apparently a complete absence of methylated products in the ether-insoluble portion.

The residues resulting from evaporation of the ethereal filtrates were carefully crystallized from a ligroin–absolute alcohol mixture. The products obtained from these crystallizations melted at 121°; mixtures of each with known samples of 1,3-dimethyluracil (m. p. 121°) melted at the same temperature, thereby establishing identity with the latter. In order to determine whether any momomethylated uracils were formed in the reaction, the mother liquors from the above described crystallizations were evaporated and the residues carefully crystallized from ligroin and absolute alcohol. Only 1,3-dimethyluracil could be obtained by this procedure. Irrespective, then, of the amount of the reagent used, diazomethane always reacts with uracil to form the 1,3-dimethyl derivative. The minimum amount necessary for complete methylation under these conditions is four moles.

Anal. (On the methylated product melting at 121°.) Calcd. for $C_6H_8O_2N_2$: N, 20.00. Found: N, 19.87.

Methylation of Thymine. 1,3-Dimethylthymine, VI.—In the experiments on the methylation of thymine, 2-g. samples of the substance were treated with 100 cc. of ethereal solutions of diazomethane (1 to 6 molecular proportions) in 250-cc. flasks equipped with air condensers. There was a steady decrease in the ether-insoluble residue attendant upon increase in the amount of diazomethane from one to six molecular proportions. In this latter case decolorization of the reagent was effected in ten days; the ether-soluble material weighed 2.56 g. and the ether-insoluble, 0.04 g. Complete methylation was accomplished under these conditions. It could also be brought about by the use of 2.25 molecular proportions of diazomethane in a closed system during continuous shaking. Under these conditions the time of methylation was materially reduced. An attempt to methylate in alcohol medium was unsuccessful as the reagent attacked the solvent too rapidly.

The melting points of the three nitrogen-methylated thymines are as follows: 1,3-dimethylthymine 153°, 1-methylthymine 202° and 3-methylthymine 280°. The tabulated results indicate in all experiments a complete absence of methylated products in the ether-insoluble residue, as this never melted below 300°, which is considerably above the melting point of either monomethylthymine. Only thymine was isolated from the ether-insoluble residue. Crystallization of the ether-soluble material from ligroin and alcohol gave in each experiment a product which was identical with 1,3-

dimethylthymine (m. p. 153°). An examination of the mother liquors from these crystallizations for monomethylated products revealed only further quantities of dimethylthymine.

Anal. (Kjeldahl). Calcd. for $C_7H_{10}O_2N_2$: N, 18.18. Found: N, 18.01, 18.02.

Methylation of 4-Methyluracil. 1,3,4-Trimethyluracil, VIII.—Two-gram samples of 4-methyluracil were treated with four molecular proportions of diazomethane in flasks provided with air condensers. After fifteen days, the 4-methyluracil had completely dissolved and there was a small residuum, which was apparently polymerized diazomethane. This was filtered off and the yellow solution evaporated to dryness. The residue thus obtained was crystallized from ligroin and absolute alcohol; it melted at 109° and was identical with 1,3,4-trimethyluracil (m. p. 109°). A typical experiment carried out under the above-described conditions gave the following results: ether-soluble material, 2.49; ether-insoluble material, 0.04 g.; m. p. of ether-soluble material, 109°.

Anal. Calcd. for $C_7H_{10}O_2N_2$: N, 18.18. Found: N, 18.01, 18.02.

The Action of Diazomethane on Cytosine.—In preliminary experiments, 2-g. samples of cytosine were placed in 250-cc. flasks and treated with quantities of diazomethane corresponding to 1, 2, 3, 4 and 5 molecular proportions. The methylation proceeded so slowly under these conditions that it became necessary to adopt a rather drastic procedure, namely, to carry out the reactions, during continuous shaking, in porcelain stoppered bottles, with large quantities of diazomethane, the reagent in certain experiments being replenished from time to time until there was no further decolorization. The data from typical experiments are given in Table II.

TABLE II

THE ACTION OF DIAZOMETHANE ON CYTOSINE

Diazomethane (mol. proportions)	7	10	13	15	15
Ether-insoluble material, g.	1.39	0.87	1.05	0.75	0.51
Ether-soluble material, g.	0.87	1.64	1.69	1.69	2.63
Time, days	17	25	4 months	4 months	33

A. A New Crystalline Form of Cytosine.—The insoluble material, after treatment with diazomethane, was filtered off and first examined for methylated cytosines by extraction with alcohol–petroleum ether. The amount of soluble material was negligible. The insoluble residue was then crystallized twice from aqueous acetone. The compound thus obtained was identical with cytosine in respect to its melting point, degree of hydration, analytical data and the melting point of its picrate. Under the microscope, however, it presented an entirely different appearance. The crystals of cytosine are normally quite platy, and occasionally of spear-shaped appearance, while the new form crystallized in peculiarly characteristic stout prisms. The two varieties melted unchanged when mixed; this was also true of their picrates. A number of attempts were made to produce this prismatic form by crystallization of ordinary cytosine under many conditions, but without success. On the other hand, when the new form was dehydrated, and then crystallized from water, or aqueous acetone, the ordinary or tabular form resulted. A comparison of the two forms is given in Table III.

TABLE III

COMPARISON OF THE TWO CRYSTALLINE FORMS OF CYTOSINE

Variety of cytosine	M. p., °C.	Hydrate, % N	Anhydrous, % N	Water, % H_2O	Picrate, m. p., °C.
Tabular (ordinary)	302–306	32.56	37.84	13.95	260–280
Prismatic (new)	306	32.60	37.70	13.69	260–280

B. **2-Oxy-6-methylaminopyrimidine, VII.**—The writers had great difficulty in extracting a definite methylation product from the dark-colored residues resulting from evaporation of the ether solutions, after the insoluble residues had been filtered off. A very small amount of the picrate of 2-oxy-6-methylaminopyrimidine was isolated by the following procedure. Ether-soluble material from the methylation experiments was acidified with hydrochloric acid and the solution decolorized as much as possible with norite. After careful evaporation to dryness, the residue was taken up in 1% hydrochloric acid and the base precipitated by the addition of picric acid. The highly colored impure picrate was crystallized several times from aqueous methyl alcohol. The small amount of crystals obtained in this manner melted at 220° and also at the same temperature when mixed with a sample of the picrate of synthetic 2-oxy-6-methylaminopyrimidine, (m. p. 220–225).[4] The former, like the latter, gave a positive test with the Wheeler–Johnson reaction,[1] and with the sulfanilic acid test,[1] thus showing the absence of a substituent in position 3.

We propose in subsequent work to accumulate sufficient ether-soluble methylated material to permit of thorough search for other methylated products.

The Action of Diazomethane on Yeast Nucleic Acid.—The yeast nucleic acid was prepared by Kowalevsky's[9] procedure. The sample isolated in this manner analyzed as follows

Nitrogen (Kjeldahl)	11.39	11.48
Phosphorus	5.97	6.25

Two ten-g. samples of the nucleic acid were placed in pressure bottles and each was treated during the course of three months with approximately 40 g. of diazomethane. The reagent was added in portions of about 4 g. whenever decolorization had occurred. The first addition of diazomethane was accompanied by vigorous evolution of nitrogen, probably by reason of its reaction with the phosphoric acid residues, but after this initial period of activity there was only gradual fading of the color. After methylation was considered to be complete, as evidenced by the persistence of the yellow color, the mixture was filtered and the filtrate evaporated to dryness. The weights of the ether-soluble portions in Samples 1 and 2 were, respectively, 5.90 and 6.80 g. and of the ether-insoluble portions, 6.0 and 7.40 g. Analysis of the ether insoluble material yielded the following results.

	Sample 1		Sample 2	
Nitrogen	8.45	8.60	9.84	9.83
Phosphorus	5.56	5.68	5.62	5.84

These data would indicate that there had been extensive combination with diazomethane. The nitrogen content decreased far more than that of phosphorus.

The soluble and the ether-soluble portions resulting from the reactions were separately hydrolyzed, but owing to the conditions (or duration) of methylation, profound changes had occurred and it was found impossible to isolate either methylated or unmethylated pyrimidines by application of one of the usual procedures.[10] A similar behavior of diazomethane was observed by Levene[11] in the methylation of xanthosine, and is well characterized in his statement to the effect that "the hydrolytic and oxidizing actions of diazomethane, and their mechanisms, deserve special investigation." This preliminary investigation has, therefore, served to emphasize the importance of using a medium and conditions for methylation which will accomplish this reaction in a shorter time and thus obviate destructive side reactions.

[9] Kowalevsky, *Z. physiol. Chem.*, **69**, 240 (1910).

[10] Johnson and Brown, *J. Biol. Chem.*, **54**, 731 (1922).

[11] Levene, *ibid.*, **55**, 437 (1923).

Summary

1. A study has been made of the action of diazomethane on the cyclic ureides, uracil, thymine, cytosine and 4-methyluracil.

2. Uracil, thymine and 4-methyluracil were readily converted into their 1,3-dimethyl derivatives by the action of diazomethane.

3. Cytosine methylated very slowly; the unmethylated portion was a new crystalline variety of this compound; the methylated portion contained among other products a small quantity of 2-oxy-6-methylaminopyrimidine, which was isolated in the form of its picrate.

NEW HAVEN, CONNECTICUT

[CONTRIBUTION NUMBER 404 FROM THE RESEARCH LABORATORY, EASTMAN KODAK COMPANY]

ACETOLYSIS OF CELLULOSE AND THE ISOLATION OF TWO CRYSTALLINE FORMS OF GLUCOSE PENTA-ACETATE[1]

BY C. S. WEBBER, C. J. STAUD AND H. LEB. GRAY

RECEIVED SEPTEMBER 5, 1929 PUBLISHED APRIL 7, 1930

Introduction

Glucose penta-acetate as an end-product of the acetolysis of cellulose is frequently mentioned: Weltzien and Singer,[2] Harold Hibbert,[3] Irvine and Soutar,[4] Freudenberg,[5] H. Ost.[6] In 1912 Klein[7] stated he believed the water-soluble products of acetolysis to be aceto-sulfates of dextrose or cellobiose. In the same year Ost[8] and Ost and Katayama[9] published two papers concerning the production of glucose penta-acetate in the acetolysis of cellulose.

The first note stated that acetolysis was carried to maximum water solubility. Ether extraction of the aqueous solution and reacetylation of the gum obtained with cold acetic anhydride and sulfuric acid yielded needles of glucose penta-acetate from alcohol. The second was concerned with the acetolysis of cellulose, hydrocellulose and alkali cellulose and in all cases after reacetylation penta-acetylglucose was obtained.

Ost in a more complete account of his work suggested that dextrose acetates are not to be looked for in the precipitate but in the ether ex-

[1] Presented before the Division of Cellulose Chemistry at the 78th Meeting of the American Chemical Society, Minneapolis, Minnesota, September 9 to 13, 1929.

[2] Weltzien and Singer, *Ann.*, **443**, 71 (1925).

[3] Hibbert, *J. Ind. Eng. Chem.*, **13**, 256 (1921).

[4] Irvine and Soutar, *J. Chem. Soc.*, **117**, 1489 (1920).

[5] Freudenberg, *Ber.*, **54**, 771 (1921).

[6] Ost, *Ann.*, **398**, 313 (1913).

[7] Klein, *Z. angew. Chem.*, **25**, 1409–1415 (1912).

[8] Ost, *Chem.-Ztg.*, **36**, 1099–1100 (1912).

[9] Ost and Katayama, *Z. angew. Chem.*, **25**, 1467 (1912).