209. *The Permanganate Oxidation of Thymine and Some 1-Substituted Thymines.*

By M. H. Benn, B. Chatamra, and A. S. Jones.

Thymine was oxidised by potassium permanganate at 37° and pH 9 to "thymine glycol." The latter was then hydrolysed and oxidised further to give urea, acetol, pyruvaldehyde, pyruvic acid, and formic acid. When the oxidation was carried out at pH 7 the degradation of the glycol was much slower than at pH 9. 1-Methyl-, 1-phenyl-, and 1-benzyl-thymine were synthesised and subjected to the same oxidation procedures. The formation of glycols from 1-substituted thymines could not be established, but *N*-substituted ureas were identified, the other oxidation products being acetol, pyruvic acid, and formic acid.

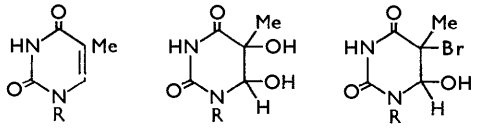
Treatment of deoxyribonucleic acid with potassium permanganate at 37° and pH 9 oxidised almost completely the cytosine, thymine, and guanine residues, but left 95% of the adenine residues unaffected.[1] In order to elucidate the nature of this reaction, simple derivatives of purines and pyrimidines have been oxidised under the same conditions. This paper reports the results obtained with thymine derivatives.

Numerous studies have been made of the action of oxidising agents, other than permanganate, on thymine. The products usually detected were urea, acetol, pyruvic acid, and formic acid.[2] Little is known about the permanganate oxidation of thymine apart from the early work of Steudel [3] who claimed to have detected the formation of urea and oxalic acid.

In the present work, oxidation of thymine (I; R = H) with potassium permanganate (1 mol.) at 37° and at pH 7 gave a glycol (II; R = H) as the major product. This was identified by comparison (m. p., infrared spectrum, and R_F value) with authentic "thymine glycol" synthesised by Baudisch and Davidson's method [4] from thymine *via* 5-bromo-5,6-dihydro-6-hydroxythymine (III; R = H). Its formation by permanganate oxidation

[1] Bayley and Jones, *Trans. Faraday Soc.*, 1959, **55**, 492.
[2] Bendich in "Nucleic Acids," Vol. I, by Chargaff and Davidson, Academic Press, 1955, p. 120.
[3] Steudel, *Z. physiol. Chem.*, 1901, **32**, 241.
[4] Baudisch and Davidson, *J. Biol. Chem.*, 1925, **64**, 233; Baudisch and Bass, *J. Amer. Chem. Soc.*, 1924, **46**, 184.

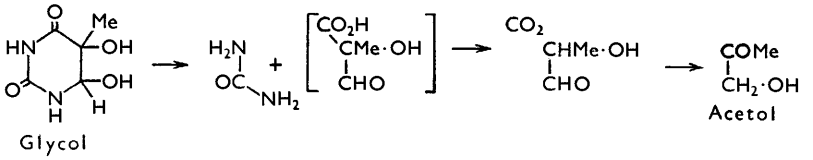
indicated that it was a *cis*-glycol. The presence of five other oxidation products including a little unchanged thymine was detected chromatographically.



(I) (II) (III)

When the oxidation was carried out at pH 9, although the same products were obtained, the amounts of these differed from those obtained by oxidation at pH 7. Thus, much more unchanged thymine was present despite the fact that all the permanganate had been consumed and thymine glycol could not be isolated and was only detectable chromatographically. The other oxidation products were identified as follows: (i) acetol, pyruvaldehyde, and pyruvic acid were identified chromatographically and by the formation of characteristic derivatives with 2,4-dinitrophenylhydrazine, (ii) formic acid was identified by reduction to formaldehyde and detection of the latter by means of chromotropic acid, and (iii) urea was detected by the use of urease and chromatographically by the use of a fructose spray. The presence of lactic and acetic acid could not be excluded, but no definite evidence of their presence was obtained.

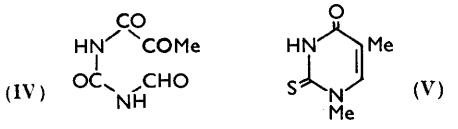
When thymine glycol was treated at pH 9 at 37° hydrolysis occurred, with the production of acetol and urea (cf. Baudisch and Davidson [4]). It appeared therefore that potassium permanganate oxidised thymine to the glycol and that the latter was then slowly hydrolysed at pH 7 and more rapidly at pH 9 to give acetol, carbon dioxide, and urea [4] (see scheme). An alternative route is that proposed by Baudisch and Davidson [4] for the oxidation of thymine with oxygen, namely, oxidation of the glycol to *N*-formyl-*N'*-pyruvylurea (IV) followed by hydrolysis to formic acid, pyruvic acid, and urea. This mechanism explains the formation of formic acid but does not account for that of acetol and pyruvaldehyde or for the higher yield of thymine glycol obtained at pH 7 than at pH 9. It is possible that the oxidation proceeds by both pathways.



Glycol Acetol

Scheme for the hydrolysis of thymine glycol.

The synthesis of 1-methylthymine was first attempted by Johnson and Clapp's method.[5] The product was found chromatographically to contain 1-methyl-2-thiothymine (V) as an impurity. This could not be removed by fractional crystallisation, but treatment



(IV) (V)

of the impure 1-methylthymine with chloroacetic acid followed by acid hydrolysis gave a pure product identical with 1-methylthymine synthesised by Shaw and Warrener's method.[6]

[5] Johnson and Clapp, *J. Biol. Chem.*, 1908, **5**, 49.
[6] Shaw and Warrener, *J.*, 1958, 153.

1-Phenylthymine (I; R = Ph) was synthesised by Shaw and Warrener's method, and 1-benzylthymine by both the above procedures.

Attempts were made to synthesise a glycol (II; R = Me) from 1-methylthymine by treatment of the bromohydrin (presumably III; R = Me), with moist silver oxide. The glycol was not isolated, however, nor could its formation be detected chromatographically, but *N*-methylurea was obtained as the product of the reaction. Treatment of 1-methylthymine bromohydrin with moist silver carbonate gave a number of products which were detected on paper chromatograms by the fructose and silver nitrate sprays. One of these products may have been the required glycol, but it was present in only small yield and could not be isolated. Oxidation of the 1-methylthymine with osmium tetroxide in hydrogen peroxide and t-butyl alcohol gave a spot on the chromatogram which corresponded with this component, but again the required glycol could not be isolated.

Oxidation of the 1-substituted thymines with potassium permanganate at 37° at pH 7 and pH 9 gave *N*-substituted ureas, acetol, pyruvic acid, and formic acid. No 1-substituted thymine glycols were isolated, although in each case a component which may have been the glycol was detected chromatographically. It was evident that if 1-substituted thymine glycols were formed they were much more unstable than thymine glycol and readily decomposed into substituted ureas.

These results suggest, therefore, that oxidation of deoxyribonucleic acids with potassium permanganate at pH 9 at 37° converts the thymine residues into ureido-residues. Other results [7] indicate that the ureido-residues would probably be stable under the conditions of the oxidation.

Experimental

Unless otherwise stated, paper chromatograms were developed by the descending method, with Whatman No. 1 paper and butan-1-ol–ethanol–water (4 : 1 : 5). The compounds were generally located by the very sensitive method of Trevelyan *et al.*[8]. This procedure is indicated here by "silver nitrate spray." Urea derivatives were located by a method based on the location of fructose with urea hydrochloride.[9] The moist paper was exposed to hydrochloric acid fumes, dried, sprayed with a dilute aqueous solution of fructose, and heated at 100° for 15 min.

"*Thymine glycol*," 4,5-*Dihydro*-4,5-*dihydroxythymine*.—This compound was prepared from thymine *via* 5-bromo-5,6-dihydro-6-hydroxythymine as described by Baudisch and Davidson [4] and was obtained as colourless prisms, m. p. 214—216° (decomp.) [lit.,[4] m. p. *ca.* 220° (decomp.)]. Paper chromatography gave a single, dark brown spot, R_F 0·28, with the silver nitrate spray. The compound reduced ammoniacal silver nitrate and Fehling's solution slowly in the cold and rapidly on heating.

Periodate oxidation. The periodate oxidation of thymine glycol (80 mg., 0·5 mmole) was examined by Jeanes and Wilham's method.[10] 0·1 mol. of periodate was consumed in the first 5 min., 0·07 mole in the second 5 min., and 0·77 mole in the third 5 min. No further uptake was observed during the next 48 hr. This induction period of 10 min. was repeatedly observed but no detailed examination of the reaction was made.

Hydrolysis. Thymine glycol (0·25 g.) was dissolved in water (10 ml.) containing sufficient sodium hydrogen carbonate to bring the pH to 9, and the mixture was kept at 37° for 19 hr.

(i) Urea was detected by the use of urease followed by the Nessler reagent, and acetol was detected by the use of *o*-aminobenzaldehyde.[11]

(ii) The mixture was acidified with 4N-hydrochloric acid and treated with excess of 2,4-dinitrophenylhydrazine in dilute hydrochloric acid. The 2,4-dinitrophenylosazone of acetol [12] was obtained. No other carbonyl compounds could be detected.

[7] Benn and Jones, *Chem. and Ind.*, 1959, 997.
[8] Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444.
[9] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[10] Jeanes and Wilham, *J. Amer. Chem. Soc.*, 1950, **72**, 2655.
[11] Baudisch, *Biochem. Z.*, 1918, **89**, 279.
[12] Bülow and Seidel, *Annalen*, 1924, **439**, 55; Neuberg and Kobel, *Biochem. Z.*, 1928, **203**, 466; Strain, *J. Amer. Chem. Soc.*, 1935, **57**, 758.

Permanganate Oxidation of Thymine.—(i) *At* pH 7. A solution of potassium permanganate (1·28 g., 8·1 mmole) in water (40 ml.) was mixed with one of thymine (1·00 g., 8·1 mmole) in phosphate buffer (pH 7), and the mixture was kept at 37° for 19 hr. The precipitated manganese dioxide was filtered off and washed with warm water, the combined filtrate and washings were treated with sufficient sodium metabisulphite to destroy the unconsumed permanganate and were then concentrated under reduced pressure at $<40°$ to *ca.* 10 ml. Thymine glycol, m. p. (on recrystallisation) 214—216° (decomp.) (0·25 g.) (Found: C, 37·2; H, 5·3; N, 17·3. Calc. for $C_5H_8N_2O_4$: C, 37·5; H, 5·0; N, 17·6%), separated as colourless crystals. A mixed m. p. with authentic thymine glycol was undepressed, the infrared spectrum in Nujol mull was superimposable on that of the authentic glycol, and in all other respects these two compounds were identical.

Paper chromatography of the mother-liquors showed that the solution contained a complex mixture similar to that obtained in the oxidation at pH 9 (see below).

(ii) *At* pH 9. A solution of potassium permanganate (2·75 g.) in water (100 ml.) was added to a solution of thymine (2·0 g.) in water (400 ml.) containing sufficient sodium hydrogen carbonate to buffer the mixture at pH 9. The mixture was treated in the way described above and concentrated to a final volume of *ca.* 20 ml., from which only inorganic salts could be isolated crystalline. Paper chromatography revealed seven compounds with the following R_F's and colours with silver nitrate spray. A, 0·01, white; B, 0·03, brown; C, 0·05, brown; D, 0·26, brown; E, 0·31, white; F, 0·36, brown; G, 0·56, pink-brown. The hydrochloric acid–fructose spray produced a single blue-black spot, R_F 0·32; ultra-violet light revealed a single spot, R_F 0·56.

Identification of the Products of Oxidation at pH 9.—Components G, D, and E were chromatographically identical with thymine, thymine glycol, and urea respectively.

Carbonyl compounds. (a) The solution containing the oxidation products (10 ml.) was made faintly acid with 4N-hydrochloric acid and treated with a solution of 2,4-dinitrophenylhydrazine in dilute hydrochloric acid until no further precipitation occurred. The crude precipitate was extracted with boiling ethanol (4 × 5 ml.), and the red crystalline residue recrystallised from nitrobenzene to give acetol 2,4-dinitrophenylosazone, red prisms, m. p. and mixed m. p. 299—300° (Found: C, 41·7; H, 2·55; N, 26·3. Calc. for $C_{15}H_{12}N_8O_8$: C, 41·7; H, 3·0; N, 25·9%). Concentration of the ethanol extracts of the crude hydrazones yielded yellow crystals which after several recrystallisations from ethanol gave yellow prisms, m. p. 218° undepressed with pyruvic acid 2,4-dinitrophenylhydrazone [13] (Found: C, 40·6; H, 3·05; N, 20·75. Calc. for $C_9H_8N_4O_6$: C, 40·3; H, 3·0; N, 20·9%), $\lambda_{max.}$ in 0·2M-aqueous sodium hydrogen carbonate 378 mμ (lit.,[13] 378—380 mμ). On paper chromatography of this substance and authentic pyruvic acid 2,4-dinitrophenylhydrazone each gave two spots, R_F 0·40 and 0·60, both yellow, the former much more intense than the other, both giving a red colour with a methanolic potassium hydroxide spray.

(*b*) The solution containing the oxidation products gave a strong positive test for acetol when treated with *o*-aminobenzaldehyde.

(*c*) The solution of oxidation products was compared chromatographically with acetol and pyruvaldehyde. The components were located by silver nitrate spray, but the chromatograms were then kept at room temperature overnight before being sprayed with ethanolic sodium hydroxide. Spots corresponding to both acetol and pyruvaldehyde were obtained, though the latter spot was barely detectable in the chromatogram of the oxidation products.

Acids. (*a*) A solution of the oxidation products (10 ml.) was run down a column of Amberlite I.R.-120 (H form) (1·5 × 25 cm.) and eluted with water until the eluates were no longer strongly acid. Paper chromatography of the eluate by the ascending method in ethanol–water–ammonia (*d* 0·88) (70 : 30 : 5·5) for 20 hr., followed by spraying the dried paper with buffered Bromophenol Blue [14] revealed two spots, one yellow and the other blue. The first spot was identical with pyruvic acid and the other corresponded to acetic, formic, or lactic acid.

(*b*) The carbonyl compounds were removed from a portion (10 ml.) of the solution of the oxidation products by treatment with 2,4-dinitrophenylhydrazine in dilute hydrochloric acid as described above. The filtrate obtained after removal of the dinitrophenylhydrazones was

[13] James and Martin, *J. Biol. Chem.*, 1952, **50**, 679.
[14] Clingman and Sutton, *Fuel*, 1952, **31**, 259.

distilled and the first 5 ml. of distillate were tested for formic and acetic acid by the methods of Eegriwe [15] and Vogel.[16] The tests were positive for formic acid but inconclusive for acetic acid.

(*c*) Comparison of the chromatograms of the solution of oxidation products with appropriate markers showed that spot A corresponded to sodium hydrogen carbonate, spot B to sodium pyruvate, and spot C to sodium formate and acetate.

1-*Methylthymine*.—(i) As prepared by the method of Shaw and Warrener,[6] 1-methylthymine was obtained as colourless needles, m. p. 288—289° (decomp.) [lit., m. p. 280—282° (decomp.),[5] 281° (decomp.) [6]]. This product was chromatographically homogeneous, R_F 0·62, and had $\lambda_{max.}$ in H_2O 272 (ε 10,000), $\lambda_{min.}$ 238 mμ (ε 1560) (Found: C, 51·6; H, 5·8; N, 20·3. Calc. for $C_6H_8N_2O_2$: C, 51·4; H, 5·75; N, 20·0%).

(ii) The preparation of 1-methylthymine was also attempted by Johnson and Clapp's method.[5] The product, m. p. 280—282°, obtained by boiling 2-ethylthio-4-hydroxy-1,5-dimethylpyrimidine with hydrobromic acid was, however, not homogeneous when examined by paper chromatography. In addition to 1-methylthymine, R_F 0·62, a compound of R_F 0·75 was detectable with ultraviolet light. The contaminant was identified as 1-methyl-2-thiothymine by chromatographic comparison with an authentic sample.[5] Repeated recrystallisation from water failed to remove this compound and purification was therefore effected as follows. The crude product (1·75 g.) was boiled under reflux with chloroacetic acid (1·50 g.) in water (20 ml.) for 2 hr. The cold solution was made faintly alkaline by addition of sodium hydroxide and then ammonia. The solution was boiled to expel excess of ammonia and allowed to cool slowly. Crystals of 1-methylthymine, m. p. 288—289° (decomp.) separated. This product was chromatographically homogeneous, and identical with the product obtained by the other route.

1-*Phenylthymine*.—This compound was obtained [6] as colourless needles, m. p. 200° (decomp.), chromatographically homogeneous (R_F 0·85), $\lambda_{max.}$ in water 275 (ε 12,000), $\lambda_{min.}$ 240 mμ (ε 4520).

1-*Benzylthymine*.—(i) *N*-Ethoxycarbonyl-β-methoxy-α-methylacrylamide [6] (5 g.) and redistilled benzylamine (3 ml.) were heated on a steam-bath for 2 hr. 2N-Aqueous sodium hydroxide (50 ml.) was added and the mixture heated for a further 10 min. with frequent shaking. The supernatant liquid was decanted, leaving a viscous syrup, and made just acid with 4N-hydrochloric acid. The precipitated solid was filtered off and recrystallised from water to give plates of 1-benzylthymine, m. p. 164—166° (decomp.) (2·17 g.) (Found: C, 67·0; H, 5·8; N, 12·8. $C_{12}H_{12}N_2O_2$ requires C, 66·65; H, 5·6; N, 12·95%). Additional material was obtained by extracting the residual syrup with ethanol and concentrating the extracts. The product was chromatographically homogeneous, R_F 0·89, had $\lambda_{max.}$ in H_2O 270 (ε 10,000), $\lambda_{min.}$ 240 mμ (ε 1930).

(ii) Benzyl chloride (12 g.) was boiled with 2-ethylthio-4-hydroxy-5-methylpyrimidine [17] (15 g.) and potassium hydroxide (4·9 g.) in ethanol (80 ml.) for 8 hr. The mixture was filtered, the residual salts were washed with ethanol, and the combined filtrate and washings concentrated to dryness under reduced pressure. The residual solid was extracted with 5% aqueous sodium hydroxide (50 ml.) and then with ether (3 × 100 ml.). The residual crystals recrystallised from benzene, to give 1-*benzyl*-2-*ethylthio*-4-*hydroxy*-5-*methylpyrimidine*, m. p. 123° (10·1 g.) (Found: N, 11·0. $C_{14}H_{16}N_2OS$ requires N, 10·8%). This product was heated with concentrated hydrochloric acid (25 ml.) until the evolution of ethanethiol ceased (*ca.* 10 hr.). The mixture was evaporated to dryness, more hydrochloric acid (25 ml.) added, and the whole evaporated to dryness. The residue was extracted with ethanol, and the extracts were filtered and concentrated to yield colourless prisms, m. p. 167—168° (decomp.) (8·31 g.). The infrared spectrum in Nujol mull was superimposable on that of the 1-benzylthymine prepared by method (i), the mixed m. p. was undepressed and in all other respects the two compounds were identical.

5-*Bromo*-5,6-*dihydro*-6-*hydroxy*-1-*methylthymine*.—1-Methylthymine (1 g.) was suspended in water (15 ml.) and bromine (0·40 ml.) added. Reaction took place rapidly in the cold and the 1-methylthymine went into solution. Excess of bromine was removed by boiling, and the solution concentrated under reduced pressure. Crystals separated and were recrystallised

[15] Eegriwe, *Z. analyt. Chem.*, 1937, **110**, 20, 22.
[16] Vogel, " Qualitative Analysis," 3rd edn., Longmans Green, London, 1947, p. 335.
[17] Vogel, " Practical Organic Chemistry," 3rd edn., Longmans Green, London, 1957, p. 894.

from water to give colourless prisms of the *bromohydrin*, m. p. 158° (decomp.) (Found: C, 30·7; H, 4·0; Br, 33·9; N, 11·6. $C_6H_9BrN_2O_3$ requires C, 30·4; H, 3·8; Br, 33·7; N, 11·8%).

Attempted Preparation of 5,6-Dihydro-5,6-dihydroxy-1-methylthymine.—(i) Moist, freshly prepared silver oxide (5 g.) was shaken with the bromohydrin (1·10 g.) in water (25 ml.) for 16 hr. The combined filtrate and washings from the silver salts were concentrated under reduced pressure at room temperature. A little crystalline material separated, having m. p. 157° undepressed by starting material. Paper chromatography of the mixture revealed that, in addition to unchanged bromohydrin, there was present methylurea, R_F 0·55, which reacted white to the silver nitrate spray and blue-black to the hydrochloric acid–fructose spray. The solution did not reduce Fehling's solution even after prolonged heating and there was no spot on the chromatogram that could be assigned to the glycol.

Replacing silver oxide by silver carbonate gave a component identical in R_F with an unidentified product of the permanganate oxidation of 1-methylthymine. No crystalline 1-methylthymine glycol could be isolated. The reaction mixture, however, reduced Fehling's solution on warming.

(ii) 1-Methylthymine (1 g.) was oxidised with potassium permanganate (1·24 g.) in pH 7 buffer under the conditions used for thymine (see above). Working up in an identical manner gave no crystalline glycol.

(iii) 1-Methylthymine (200 mg.) was dissolved in 6% hydrogen peroxide in dry t-butyl alcohol [17] (30 ml.), osmium tetroxide (15 mg.) added, and the mixture kept at 25° for 24 hr. After filtration and concentration under reduced pressure, final traces of the alcohol and peroxide were removed by adding water (20 ml.) and again concentrating under reduced pressure at room temperature. A little 1-methylthymine separated, having m. p. and mixed m. p. 288° (decomp.). The solution reduced Fehling's solution fairly rapidly in the cold, but only slowly gave a faint colour with Schiff's reagent. The solution (1 ml.) was treated with aqueous 0·2M-sodium metaperiodate (2 ml.) and after 10 min. with Schiff's reagent. A strong colour was immediately produced. A control, in which water replaced the solution of reaction products, gave a negligible colour. Paper chromatography of the reaction mixture followed by location of the components with silver nitrate spray revealed several faint white spots, one of which corresponded with unchanged 1-methylthymine, and an intense brown spot R_F 0·55. No crystalline glycol could be obtained.

An attempt was made to hydroxylate 1-methylthymine (300 mg.) with osmium tetroxide (540 mg.) in dioxan (200 ml.). After 5 days at room temperature the starting material was recovered in nearly quantitative yield.

Permanganate Oxidation of 1-Substituted Thymines at pH 9.—(i) *1-Methylthymine.* 1-Methylthymine (1 g., 7·2 mmoles) was oxidised with potassium permanganate (1·25 g., 7·9 mmoles) in sodium hydrogen carbonate buffer (200 ml. of pH 9) under the conditions, and with the working up, described for thymine. Paper chromatography of the solution containing the products which reduced warm Fehling's solution revealed 6 spots with the following R_F's and colours with silver nitrate spray; A, 0·00, white; B, 0·04, brown; C, 0·09, brown; D, 0·42, white; E, 0·55, brown; F, 0·62, faint white. The procedures described previously identified: 1-methylthymine, spot F; formic acid (sodium formate), spot B; and sodium hydrogen carbonate, spot A. Acetol and pyruvic acid were found to be present, but urea was absent. *N*-Methylurea was detected as follows: The chromatogram was treated with the hydrochloric acid–fructose spray. A single blue-black spot, R_F 0·55 (spot E), was obtained, identical in R_F with *N*-methylurea. The latter, however, unlike spot *E*, gave a white spot with silver nitrate spray. Paper chromatography of the reaction mixture in propan-1-ol–water (6 : 4) resulted in partial separation of spot E into two spots, one white and the other, slower-running, brown to the silver nitrate spray. Complete resolution was obtained as follows. The solution containing the oxidation products was run down a column of Zeo-Karb 225 (H form) ion-exchange resin (1·5 × 25 cm.), and eluted with water; the eluates were collected until no longer acid, concentrated under reduced pressure at room temperature, and examined by paper chromatography. Spot E was now white to silver nitrate spray and blue-black to hydrochloric acid–fructose and chromatographically identical with *N*-methylurea. Further washing of the ion-exchange column with water and concentration of the eluates as before gave a material in the paper chromatogram of which spot E appeared as a dark brown spot with silver nitrate spray. This other component of spot E, from the nature of its reaction with the silver nitrate, appeared possibly to be 1-methylthymine glycol. It corresponded in R_F value with one product from the

action of silver carbonate on 5-bromo-5,6-dihydro-6-hydroxy-1-methylthymine, and from the hydrogen peroxide osmium tetroxide oxidation of 1-methylthymine.

(ii) Oxidation of 1-benzyl- and 1-phenyl-thymine similarly gave mixtures. By the use of paper chromatography, sodium formate, sodium pyruvate, the *N*-substituted urea, and a little unchanged 1-substituted thymine were detected. Acetol and pyruvic acid were also found. A compound which may have been the 1-substituted thymine glycol was detected on chromatograms but no crystalline glycol was isolated.

The authors thank Professor M. Stacey, F.R.S., for his interest in this work.

CHEMISTRY DEPARTMENT, THE UNIVERSITY, EDGBASTON,
BIRMINGHAM, 15.

[*Received, July 8th,* 1959.]