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THE REACTION OF PHENYL ISOCYANATE WITH PURINES, PYRIMIDINES AND DEOXYRIBONUCLEIC ACID

A. S. JONES and J. H. WARREN
Chemistry Department, The University of Birmingham, Birmingham 15

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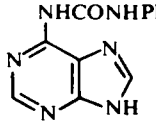
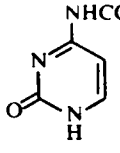
Abstract—Reaction of phenyl isocyanate with cytosine, adenine and guanine gave 4-N-phenylcarbamylcytosine, 6-N-phenylcarbamyladenine and 2-N-phenylcarbamylguanine respectively. Uracil did not react. Reaction of DNA (as the Cetavlon salt) with phenyl isocyanate at 0–4° for 10 days resulted in the almost complete substitution of the cytosine, adenine and guanine residues. From the analytical results and the fact that 3′,5′-O-diacetylthymidine did not react it was concluded that the thymine residues were unaffected.

THE chemical modification of the base residues in DNA has been studied mainly to elucidate the mode of action of mutagenic agents[1] and to determine the distribution of nucleotides. For the latter two approaches have been investigated; (a) conversion of the base into a derivative which can be readily removed and so rendering the adjacent phosphodiester linkages labile[2]; (b) reaction of one base specifically with a reagent that can be seen in the electron microscope.[3] A third approach, namely modification of a base so as to render an enzyme more selective, has been applied to tRNA.[4] This paper reports the modification of the base residues of DNA by reaction with phenyl isocyanate.

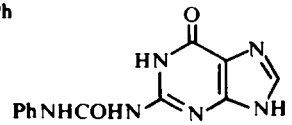
Initially, study was made of the reaction of phenyl isocyanate with purines and pyrimidines. A previous worker has shown that a reaction occurs with adenine to give a monosubstituted product which was claimed to be 6-N-phenylcarbamyl-adenine (I), but no evidence was given to support this.[5] Other workers have shown that uracil does not react with phenyl isocyanate and that cytosine forms a monosubstituted product which on the basis of its IR spectrum was considered to be 4-N-phenylcarbamylcytosine (II) but again the product was not completely characterized.[6] We have confirmed that uracil does not react even at 150°. At the same temperature cytosine gave a monosubstituted product which had a UV spectrum similar to 4-N-acylated cytosines, and which upon alkaline hydrolysis gave cytosine but upon acid hydrolysis gave uracil and phenylurea. It was concluded therefore that the compound was 4-N-phenylcarbamylcytosine (II).



I



II



III

Reaction of adenine with an excess of phenyl isocyanate at 150° in pyridine gave three products but reaction at 100° with 1 molecular proportion gave one major product which analysed correctly for a monosubstituted compound. On the basis of its UV spectrum and its occurrence in the hydrolysate of phenyl isocyanate-treated DNA it was concluded that the compound was 6-N-phenylcarbamyladenine (I). Guanine was treated with phenyl isocyanate in boiling DMF. The product analysed correctly for a monosubstituted derivative. Hydrolysis with dilute acid gave, in addition to guanine, a small amount of xanthine. This and the fact that it occurs in the acid hydrolysate of phenyl isocyanate treated DNA shows that it is 2-N-phenylcarbamylguanine (III).

The reaction of DNA with phenyl isocyanate was carried out at 0–4° for 10 days on the cetyltrimethylammonium salt in DMF and the product isolated as a white high-molecular weight solid (IV). Hydrolysis of IV with 67% formic acid at 37° for 18 hr (conditions which convert DNA into free purines and apurinic acid[7]) gave components, which upon paper chromatography had identical R_f values in two solvent systems and UV absorption spectra in acid and alkali identical with those of 6-N-phenylcarbamyladenine (I) and 2-N-phenylcarbamylguanine (III). In addition a small amount of guanine and a trace of adenine were produced, but control experiments showed that these were formed by the action of the formic acid on the phenylcarbamyl derivatives. It was concluded, therefore, that the purines of the DNA had been completely substituted. The recovery of I plus the trace of adenine and of III plus the small amount of guanine corresponded to only 73% of the adenine and 47% of the guanine, of the original DNA respectively. This low recovery was due to incomplete elution from the paper chromatograms because the total recovery of UV-absorbing material (purine plus pyrimidine derivatives) was only 84%. There were no components running with R_f values greater than I so it appeared that no disubstituted purines had been formed.

The oligonucleotide fraction remaining after the formic acid treatment had a high absorption in the region 310–320 nm. This indicated the presence of phenylcarbamylcytosine residues, but a direct measure of these was not obtained. However, measurement of the total number of phenylcarbamyl groups in IV showed that there were present 2·76 groups per 4 g atoms of phosphorus. This corresponds closely with the total number of adenine, cytosine and guanine residues, and as it could be concluded that the thymine residues did not react with phenyl isocyanate (3′,5′-O-diacetylthymidine did not react under the same conditions), the cytosine residues must also have been completely substituted.

This modification of DNA by phenyl isocyanate takes place under extremely mild conditions so that scission of phosphodiester linSages must be very small, and it is specific for the adenine, guanine and cytosine residues. It might be of potential use, therefore, for the determination of nucleotide sequence by increasing the specificity of action of nucleases. There is also the possibility that reaction of DNA with a suitable isocyanate might render the thymine residues "visible" in the electron microscope by contrast with larger substituted residues of the other bases.

EXPERIMENTAL

Paper Chromatography was carried out in the following solvents: solvent 1, propan-2-ol : ammonia (d, 0·88); water (85 : 1·3 : 15): solvent 2, propan-2-ol : 10N HCl : water (17 : 4·1 : 3·9).

4-N-Phenylcarbamylcytosine. Cytosine hydrochloride (200 mg) and phenyl isocyanate (2 ml) were heated together at 150° for 10 min. The solid which formed was filtered off and washed with light petroleum (40–60°) and then with ether. It was purified by dissolving in N KOH (10 ml) and reprecipitating (twice) with dilute HCl. It was finally washed well with water and dried *in vacuo* over phosphoric anhydride to give 4-N-*phenylcarbamylcytosine* (260 mg). (Found: C, 56·9; H, 4·2; N, 24·7. $C_{11}H_{10}N_4O_2$ requires: C, 57·2; H, 4·4; N, 24·4%); λ_{max} 294·5 nm at pH 13 (ε, 15·9 × 10^3), 303 nm at pH 1 (ε, 19·2 × 10^3). The compound was chromatographically homogeneous in solvent 1. It was very sparingly soluble in water, soluble in dilute acids and alkalis, insoluble in all common organic solvents except DMF. Hydrolysis with boiling N KOH for 30 min gave almost complete conversion into cytosine. Hydrolysis with N HCl at 100° gave uracil and phenylurea.

6-N-*Phenylcarbamyladenine.* Adenine (500 mg), phenyl isocyanate (0·46 ml) and dry pyridine (6 ml) were heated to 100° for 1 hr. Upon cooling, the mixture set to a gelatinous solid which was triturated with light petroleum (40–60°), filtered off and washed with ether. The solid residue was dissolved in pyridine, the soln filtered and the product precipitated from the filtrate by the addition of a large excess of light petroleum. Examination of the product by paper chromatography showed that it was homogeneous except for the presence of adenine. This was removed by extracting twice with boiling water (30 ml) for 1 hr. The product was filtered off and dried *in vacuo* to give 6-N-*phenylcarbamyladenine* (300 mg) (m.p. 320°d). (Found: 56·1; H, 4·0; N, 33·7. $C_{12}H_{10}N_6O$ requires; C, 56·8; H, 3·9; N, 33·1%); λ_{max} 283 nm at pH 13 (ε, 20·0 × 10^3), 286 nm at pH 1 (ε, 24·6 × 10^3). The compound was very sparingly soluble in water, soluble in dilute acids and alkalis, pyridine and DMF, insoluble in other common organic solvents.

2-N-*Phenylcarbamylguanine.* Guanine hydrochloride (500 mg), dried at 110° *in vacuo,* was dissolved in dry DMF (10 ml), phenyl isocyanate (0·5 ml) added and the mixture boiled under reflux for 10 min. The white ppt which formed was filtered off, washed with EtOH and then with ether. The resulting solid was purified by dissolving in N KOH (20 ml) and reprecipitating (twice) with N HCl. The resulting solid was centrifuged off, washed well with water and dried *in vacuo* to give 2-N-*phenylcarbamylguanine* (100mg). (Found: C, 53·1; H, 3·8; N, 30·7. $C_{12}H_{10}N_6O_2$ requires: C, 53·3; H, 3·7; N, 31·1%); λ_{max} 270 nm (ε, 18·1 = 10^3) and 239 nm (ε, 21·4 × 10^3) at pH 13 and 263 nm (ε, 27·3 × 10^3) at pH 1. The compound, which was homogeneous by paper chromatography, was insoluble in water and all common organic solvents except DMF in which it was sparingly soluble. It was slightly soluble in dilute acids and alkalis. Hydrolysis with N HCl at 100° for 30 min gave guanine and a trace of xanthine.

Treatment of 3′, 5′-O-*Diacetylthymidine with phenyl isocyanate.* 3′,5′-O-Diacetylthymidine (103 mg) was dissolved in dry DMF (10 ml), the soln heated to 40°, phenyl isocyanate (1 ml) added, the soln cooled to 4° and then left at this temp for 10 days. EtOH (2 ml) was then added and the soln allowed to stand overnight. It was then examined by tlc on silica gel using 4 elutions with $CHCl_3$. The uv-absorbing spot corresponding to the starting material was eluted and its UV absorption spectrum measured. It was identical with that of the starting material both in acid and in alkali. The recovery of 3′, 5′-O-diacetylthymidine was 98%.

Reaction of deoxyribonucleic acid with phenyl isocyanate. The Cetavlon salt of calf thymus DNA was prepared by adding aqueous Cetavlon (I.C.I. Ltd., Pharmaceuticals Division) (a mixture of long chain alkytrimethylammonium bromides, mainly the tetradecyl derivative) soln (20% w/v) to an aqueous soln of DNA until precipitation was complete. The resulting fibrous ppt was removed, washed with water, dialysed exhaustively (as a suspension) against distilled water and freeze-dried.

Cetavlon DNA (250 mg; dried *in vacuo* at 110° for 3 hr over P_2O_5) was dissolved in DMF (20 ml) by warming at 40°. Phenyl isocyanate (2 ml) was added and the soln allowed to stand at 0–4° for 10 days. It was then diluted 3 times with EtOH and dialysed against EtOH for 20hr. The non-diffusible material was then poured into 0·5 vol of M NaCl aq and the resulting ppt centrifuged off. This was dissolved in water (100 ml) and the aqueous soln was exhaustively dialysed against water for 48 hr and then freeze-dried to give a fibrous white solid (168 mg). (Found (after correction for 15% water) P, 7·2%. Theoretical (assuming P content of DNA to be 9·3% and the presence of 2·76 phenylcarbamyl groups per 4 g atom P) P, 7·5%).

Analysis of phenyl isocyanate-treated DNA

(i) *Total purines and pyrimidines.* The compound was hydrolysed with 98% formic acid at 175° for 60 min and the free purines and pyrimidines so formed separated by paper chromatography in solvent 2 and determined spectrophotometrically. Within experimental error the base composition was identical with that of the original DNA (43% G + C).

(ii) *Total phenylcarbamyl groups.* The compound (10 mg) was boiled under reflux with 0·3N KOH (2 ml) for 30 min and then the contents distilled into a 25 ml graduated flask. 2 ml quantities of water were repeatedly added to the reaction vessel and then distillation continued. The receiver was finally made up to the mark and the aniline content measured spectrophotometrically at 280 nm. IV contained 2·76 phenylcarbamyl groups per 4 g atoms of P. This method gave, with 6-N-phenylcarbamylcytosine, results which were within 2% of the theoretical values. It was also shown that the substituted purines were completely converted into the unsubstituted base and aniline by this procedure.

(iii) *Phenylcarbamyl substituted products.* The phenyl isocyanate treated DNA (3·7 mg) was dissolved in 98% formic acid (1ml) and water (0·5 ml) added. The resulting suspension was allowed to stand at 37° for 23 hr after which a clear soln was obtained. Samples of the soln were subjected to paper chromatography on Whatman No. 1 paper in solvent 2. Spots corresponding with markers of adenine (R_f 0·52) guanine, (R_f 0·3), 6-N-phenylcarbamyladenine (R_f 0·8) and 2-N-phenylcarbamylguanine (R_f 0·55) were obtained. These spots were eluted with 0·1 N NaOH and their UV absorption spectra determined, which corresponded with those of the markers both in acid and in alkali. Because of the close proximity of the adenine and the 2-N-phenylcarbamylguanine, for quantitative determination they were eluted together and the amount of the latter determined by measuring the change in absorption at 235nm on changing from acid to alkali. The amount of adenine was then obtained by difference. The following recoveries were obtained (moles of base/4 g atoms P): 6-N-phenylcarbamyladenine, 0·795; adenine, 0·014; 2-N-phenylcarbamylguanine, 0·234; guanine, 0·182. In addition there was considerable UV-absorbing material at the origin and streaking to just behind the guanine spot. This was eluted and found to have a peak at 268 nm and a pronounced shoulder at 300–320 nm. The whole of the chromatography paper was eluted with 0·1N NaOH but the total recovery of UV-absorbing material was only 84%.

Hydrolysis of 6-N-phenylcarbamyladenine under similar conditions gave 5% conversion into adenine; with 2-N-phenylcarbamyl-guanine, a 20% conversion into guanine was obtained.

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