hydride in mineral oil dispersion with dry hexane) in 120 ml of dimethyl sulfoxide was heated at 50° until solution took place (about 30 min). After addition of 22.6 g (0.063 mole) of triphenylmethylphosphonium bromide the reaction was heated for 3 hr at 70°. A solution of 2.00 g (0.0063 mole) of XI in 120 ml of tetrahydrofuran was added and the reaction was heated overnight under reflux. The cooled reaction was poured into a mixture of ice and water containing 40 g of sodium bicarbonate. This was extracted with benzene and the extracts were dried (Na_2SO_4) and concentrated to a semicrystalline residue. Addition of ether and filtration gave two crops of triphenylphosphine oxide which contained no XXI by thin layer chromatography and were discarded. The evaporated mother liquors were passed over silica gel in 1:1 hexane–benzene. The eluates were concentrated and crystallized from hexane at Dry Ice temperature to give 0.94 g (47%) of XXI as tan crystals, mp 35–50°. Further recrystallization gave the analytical sample as colorless crystals: mp 48–52°; $\lambda_{max}^{C_2H_5OH}$ 230 mμ (infl) (ε 9500), 277 (1200), 284 (1230); nmr, see Table I.

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 80.26; H, 9.21.

***dl*-6aβ,7,8,9,10,10aβ-Hexahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[*b*,*d*]pyran-1,9-diol (XXII).** A slurry of 500 mg (1.58 mmoles) of XI in 10 ml of ether was added to a solution of methylmagnesium iodide prepared from 106 mg (4.4 mg-atoms) of magnesium and 0.30 ml (0.68 g, 4.8 mmoles) of methyl iodide in 10 ml of ether to give a clear reaction mixture. After heating for 1 hr under reflux the reaction was cooled in an ice bath and 1 *N* hydrochloric acid was added until two clear layers were formed. The organic layer was diluted with ether and washed twice with water, once with dilute sodium bicarbonate, and again with water. The ether solution was dried (Na_2SO_4) and concentrated to an oil which on crystallization from hexane gave 380 mg (72%) of XXII as colorless crystals, mp 142–145°. Recrystallization from ether–hexane gave the analytical sample: mp 142–145°; $\lambda_{max}^{C_2H_5OH}$ 230 mμ (infl) (ε 10,000), 278 (1420), 283 (1450).

Anal. Calcd for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 76.07; H, 9.83.

***dl*-6aβ,7,8,10aβ-Tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[*b*,*d*]pyran-1-ol (XXIII).** A solution of 828 mg (2.5 mmoles) of XXII and 50 mg of *p*-toluenesulfonic acid in 50 ml of benzene was heated for 30 min under reflux. The cooled solution was washed with dilute sodium bicarbonate, dried (Na_2SO_4), and evaporated to give 770 mg (98%) of XXIII as a colorless oil. A small portion of this oil was evaporatively distilled at 135° (0.05 mm) to give the analytical sample: $\lambda_{max}^{C_2H_5OH}$ 232 mμ (infl) (ε 9850), 275 (1470), 282 (1500); nmr, see Table I.

Anal. Calcd for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62. Found: C, 79.87; H, 9.47.

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Photochemistry of Nucleic Acids. III. The Structure of DNA-Derived Thymine Photodimer[1]

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Contribution from the University Chemical Laboratory, Lensfield Road, Cambridge, England. *Received June 29, 1967*

Abstract: The identity of the major thymine photoproduct derived from DNA is established with the *cis–syn* dimer **1** obtained from irradiation of thymine in ice. The ^{14}C-labeled photoproduct was mixed with unlabeled ice-dimer and the cocrystallized material treated with bromine in alkali. The resulting stereospecific rearrangement reaction gave *cis,cis,cis*-3-carbonamido-1,7-dimethyl-3,5,0-triazatricyclo[5.3.0.$0^{2,6}$]deca-4,8,10-trione (**7**) retaining more than 91% of the specific activity of the recrystallized dimer. The mechanism of the rearrangement was investigated by performing the reaction in oxygen-18-enriched solvent. The observed nonincorporation of this isotope into the product **7** is consistent with a mechanism involving isocyanate formation without nucleophilic attack of hydroxide.

Good evidence exists[3,4] that photoproducts of thymine and of cytosine are responsible for the majority of photobiological effects caused by ultraviolet irradiation of DNA. The formation of pyrimidine dimers is particularly important in this respect and, in principle, they can arise either by photoaddition of adjacent pyrimidines in the same DNA strand–intrastrand dimerization or from bonding between two neighboring pyrimidines, one in each strand, giving interstrand dimers and thus effecting cross-linking between the complementary DNA chains.

These two modes are expected to lead to dimers of different stereochemistry, and the inspection of molecular models resulted in the prediction[5] that intrastrand dimers should have *cis–syn* stereochemistry whereas interstrand dimers would give rise to either *cis–anti* or to *trans–anti* products. An alternative opinion[6] has suggested that such cross-linking would afford *cis–anti* or *trans–syn* interstrand dimers.

(1) Paper II: G. M. Blackburn and R. J. H. Davies, *J. Chem. Soc., C*, 2239 (1966).

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(3) Abbreviations used: DNA, deoxyribonucleic acid; t-RNA, transfer ribonucleic acid; T, thymine; TT, thymine dimer; UT, uracil–thymine dimer.

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Studies of photodimerization in the solid state[7,8] have clearly demonstrated that photoaddition of two unsaturated molecules can occur only if they are initially located in suitable proximity in the crystal lattice. The same restrictions may be reasonably applied to dimerization in native DNA and in other ordered nucleic acid structures such as t-RNA. It follows that the formation of interstrand dimers would require gross distortion of the helical structure to permit pyrimidine bases in the complementary strands to approach within the limiting reaction distance (probably about 4 A). Thus interstrand dimerization appears to be an extremely improbable event in native DNA. In contrast, the formation of intrastrand dimers[9] requires only a small perturbation of the helical structure and they may be expected to predominate among the photoproducts produced in DNA.

Evidence for thymine dimerization in irradiated DNA was first obtained by Beukers,[10] later to be substantiated by Wacker[11] who achieved a separation of the various photoproducts by ion-exchange chromatography. The major DNA-derived thymine photoproduct has long been assumed to have the same structure as that isomer[12] of thymine dimer obtained by irradiation of frozen, aqueous solutions of thymine because the two have identical chromatographic properties.[13-15] However, the determination of the structure of thymine ice-dimer by reactions of defined stereochemistry[1] has provided the means of establishing a rigorous, chemical proof[16] of the structure of the major DNA-derived thymine photoproduct.

This thymine dimer has recently been isolated in milligram quantities from ultraviolet-irradiated DNA and shown to have an infrared spectrum identical with that of the ice-dimer.[17,18]

Experimental Section

Labeled DNA's. *E. coli* 15T$^-$ bacteria were grown in an ammonium salts-glucose medium supplemented with [2-^{14}C]thymine (0.25 mcurie) to give 0.4 g of wet cells and the DNA was isolated by Marmur's procedure.[19] Similarly, [6-^{3}H]thymine-labeled DNA was obtained from a thymidine-requiring mutant of *E. coli* grown in a minimal medium supplemented with [6-^{3}H]thymidine (1 mcurie; Radiochemical Center, Amersham, England).

Ultraviolet Irradiation. The DNA preparations were dissolved in dilute saline-citrate buffer (0.015 *M* NaCl, 0.015 *M* citrate) to give solutions of 0.9 mg/ml (A_{260} = 21.6) and irradiated in 1-cm quartz cuvettes. ^{14}C-Labeled DNA was exposed to light from a low-pressure mercury resonance lamp with a point source, Thermal Syndicate Ltd., No. T/M5/544A, which emitted more than 90% of its energy at 254 mμ. Wavelengths below 240 mμ were cut out by a 5-mm filter of 20% aqueous acetic acid. The total incident dose given at room temperature was estimated at 1.6 × 10^6 ergs/mm^2. The ^{3}H-labeled DNA was irradiated at some distance from a Phillips 500-w mercury are. The incident light was filtered by passage through three 1-cm cells containing water, saturated nickel sulfate solution, and carbon tetrachloride, respectively, to restrict transmission to the 260-350-mμ band. The incident dose from this source was estimated at 4 × 10^6 ergs/mm^2 after calibration of the lamp by uranyl oxalate actinometry.[20] Both DNA solutions were stirred occasionally during irradiation.

Isolation of Photoproducts. In each case the DNA was recovered from the irradiated solution by precipitation with ethanol in the usual way and was hydrolyzed by heating in solution in trifluoroacetic acid (1.5 ml) in a sealed tube at 180° for 90 min. The residues obtained after lyophilization of these solutions were dissolved in water (0.3 ml), banded on sheets of Whatman No. 1 paper, and chromatographed (ascending) with 1-butanol–acetic acid–water (80:12:30).[21] Distribution of radioactivity was determined by cutting 1-cm strips at 5-mm intervals from the base line (corresponding to successive increments of about R_f 0.02).

Samples from ^{3}H-labeled DNA were monitored using a Nuclear Chicago liquid scintillation counter. The chromatogram showed three major peaks at R_f 0.64, 0.32, and 0.24 corresponding to thymine, thymine dimer, and uracil-thymine dimer, respectively. The ratio of TT to UT was 7:2 and these products constituted 2.6% of total thymine.

Samples from ^{14}C-labeled DNA were counted by liquid scintillation using a Nuclear Enterprises (Edinburgh) shielded scintillation head unit, NE 5503, on equipment adjusted for ^{14}C-scintillation counting. The chromatogram (Figure 1) showed three major peaks at R_f 0.64, 0.32, and 0.24. The radioactivity in the latter two areas constituted 7.6% of the total and was distributed in the ratio 7:1.

Identification of the Major DNA Photoproduct. The strip of the chromatograms corresponding with the major ^{14}C-labeled photoproduct (R_f 0.27–0.44) was excised and eluted with hot water. Unlabeled thymine ice-dimer[12] (350 mg) was added and the solution filtered and cooled. The crystalline dimer was collected, washed, and dried (305 mg) and its specific activity, 1377 ± 51 counts mg^{-1} min^{-1} (six samples), determined by counting of weighed samples adsorbed onto filter paper disks from solution in trifluoroacetic acid. A portion of the material was recrystallized from water and the specific activity found to be 1344 ± 60 counts mg^{-1} min^{-1} (six samples).

The remainder of the dimer (246 mg) was converted into the rearrangement product by treatment with sodium hydroxide and bromine in the usual way.[1] The recrystallized product (40 mg, 17%) had specific activity 1254 ± 54 counts mg^{-1} min^{-1} (eight samples). The infrared spectra of the ^{14}C-labeled dimer and of the rearrangement product were identical with those of authentic samples.

The corresponding specific activities for the ^{3}H-labeled thymine dimer (154 mg) were 1168 ± 72 counts mg^{-1} min^{-1} (three samples) and 983 ± 37 counts mg^{-1} min^{-1} after recrystallization. The rearrangement product had specific activity 690 ± 30 counts mg^{-1} min^{-1} (two samples).

In a control experiment, [2-^{14}C]thymine was converted into dimer by irradiation in ice to give a product of specific activity 1470 ± 40 counts mg^{-1} min^{-1} which was converted into the rearrangement product by bromine and alkali, specific activity 1520 ± 100 counts mg^{-1} min^{-1}.

Formation of *cis,cis,cis*-3-Carbonamido-1,7-dimethyl-3,5,9-triazatricyclo[5.3.0.0^{2,6}]deca-4,8,10-trione (7) in $D_2{}^{18}O$. A solution of Na^{18}OD was prepared by carefully dissolving sodium (400 mg) in $D_2{}^{18}O$ (2 g; 22 mole % ^{18}O). Thymine ice-dimer[12] (504 mg) was added and stirred to give a slurry of the disodium salt.[1] A sample was removed, washed with organic solvents, dried, and identified by its infrared spectrum. It was reconverted into thymine dimer by treatment with cold hydrochloric acid, recrystallized from water, and examined by mass spectrometry.

The remainder of the disodium salt suspension was cooled to 0° and bromine (0.26 ml, *ca.* 3 moles) added slowly with shaking. The product was isolated as usual, recrystallized from water, and identified as *cis,cis,cis*-3-carbonamido-1,7-dimethyl-3,5,9-triazatricyclo-[5.3.0.0^{2,6}]deca-4,8,10-trione by its infrared spectrum (76 mg; 15% yield). Pyrolysis of this compound at 240° *in vacuo* gave α,α′-dimethylmaleimide and 2-imidazolone as previously described.[1] These products were twice purified by sublimation, identified spectroscopically, and submitted to mass spectral analysis.

The mass spectra of all three products were obtained using an AEI MS9 spectrometer. The spectrum of the thymine dimer ob-

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tained from its disodium salt was identical with that of a control sample of thymine dimer run consecutively, terminating at *m/e* 126 and showing no increase in intensity at *m/e* 128 and 130. Similarly, the spectra of dimethylmaleimide and of 2-imidazolone were identical with those of authentic samples. Neither the (M + 2) peak of either sample nor the (M + 4) peak of dimethylmaleimide showed any increase in intensity.

A control experiment using D_2O not enriched with oxygen-18 gave identical results, the mass spectra of the products being identical with those of synthetical compounds.

Results and Discussion

DNA-Derived Photoproduct. The distribution of radioactivity along the chromatogram of the ^{14}C-labeled hydrolysis product of irradiated DNA (Figure 1) shows the presence of only two major photoproducts and thymine, and is similar to that obtained by Setlow and Carrier[4] in a related system. In comparable experiments, Wang[22] reported the presence of two additional radioactive areas equivalent to 13% of total thymine at R_f 0.37 and 0.45. While small peaks can be seen in these positions (Figure 1), their intensities are very low as also observed by Smith,[23] and make them of minor significance. The two dominant photoproducts are properly identified[4] as thymine dimer and uracil–thymine dimer, this latter arising by deamination[24] of a cytosine–thymine dimer.

The 7:1 ratio of TT to UT observed in the ^{14}C experiment is twice that observed in the ^{3}H experiment. This difference most probably is a consequence of the use of different irradiation sources in the two experiments. While the source used in the carbon-14 case emitted most of its energy at 254 mμ, the source for the tritium reaction gave more of its energy at longer wavelengths. The data reported by Setlow[4] show that the ratio of TT to UT can vary with the wavelength of irradiation as well as with changes in pH, in the physical state of the DNA, and in the extent of photoproduct formation.

The photoproduct eluted from the area of the chromatograms corresponding to TT gave insufficient material for a direct chemical manipulation and necessitated the addition of unlabeled carrier. The choice of thymine ice–dimer for this purpose has the unique advantage that it can be isomerized in a reaction which discriminates exclusively for a dimer of *cis–syn* stereochemistry.[1] Accordingly the two samples of DNA photoproduct were separately admixed with nonradioactive, recrystallized *cis–syn* thymine dimer and twice crystallized from water.

The results show (Table I) a small change in specific activity on recrystallization of the tritium-labeled photoproduct but no significant loss from the carbon-14-labeled thymine dimer. Both systems exhibit some change in specific activity of dimer following its rearrangement into the isomeric product 7, though the loss in the ^{14}C experiment is much lower than the diminution in specific activity in the tritium-labeled system.

The data from the ^{14}C-labeled thymine experiment unequivocally prove that at least 91% of the DNA-derived thymine photoproducts, having R_f 0.27–0.44, and cocrystallizing with *cis–syn* thymine dimer, are identical

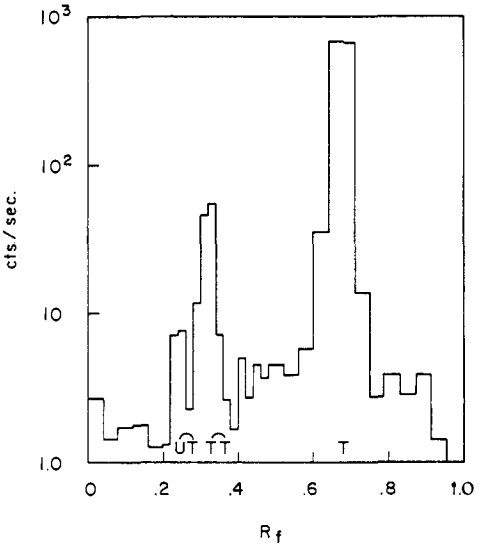

Figure 1. Chromatographic distribution of radioactive photoproducts from acid hydrolysate of ultraviolet-irradiated, ^{14}C-labeled DNA.

with this compound. The nature of the other material, comprising 9% of the total counts, is unknown, but it seems reasonable to suppose that it corresponds to the minor photoproduct which Wang[17,25] has shown to cochromatograph and cocrystallize with the dominant thymine photoproduct.

Table I. Radioactivity of Photoproduct Admixed with Thymine Ice–Dimer

	[6-^{3}H]-Thymine	[2-^{14}C]-Thymine
Product after first crystallization	1168 ± 72[a]	1377 ± 51[a]
Product after second crystallization	983 ± 37	1344 ± 60
Product after rearrangement (7)	690 ± 30	1254 ± 54

[a] Specific activities in counts mg^{-1} min^{-1}.

While the results of the tritium-labeled experiment as previously reported point to the same broad conclusion, it is clear that in this case the losses in specific activity are much larger both for the recrystallization and for the isomerization steps. They may be a consequence of one or more of the following three possibilities. Firstly, the differences in the ultraviolet-irradiation systems used in the two experiments could lead to different ratios of photoproducts.[4] Secondly, tritium may have been lost by exchange with solvent during recrystallization and/or isomerization of thymine photoproducts. Thirdly, it is possible that the relative efficiency of ^{3}H counting was lower for the solid samples of the rearrangement product than for the thymine dimer. The relative importance of these three factors cannot be assessed from the available information.

This quantitative demonstration that over 90% of the DNA-derived thymine photoproducts which cocrystallize with TT is the *cis–syn* thymine dimer is consistent with the conclusion that intrastrand dimerization

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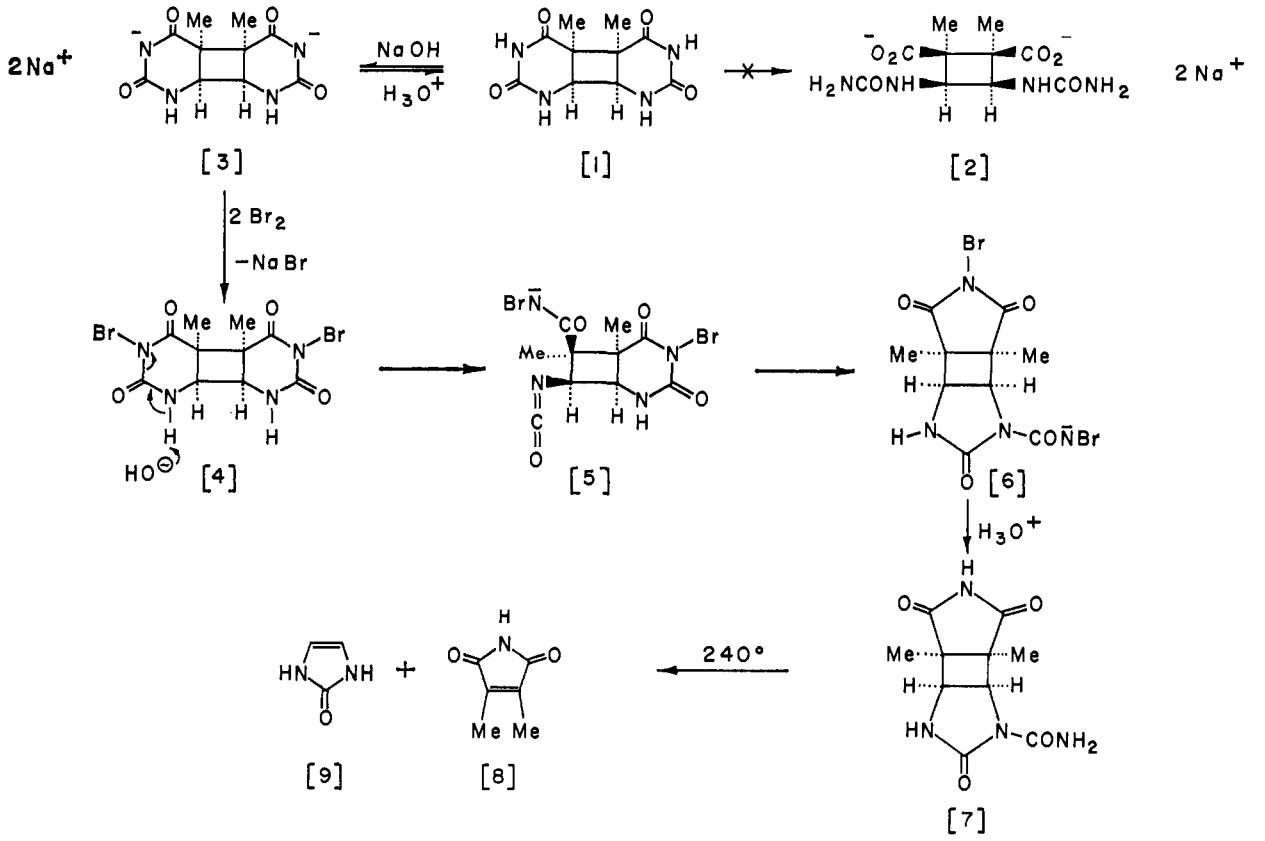

is the principal photochemical event. The same conclusion may also hold for the formation of mixed thymine–cytosine dimers since it has been argued[18] that the resulting uracil–thymine dimer has the same stereochemistry as the thymine homodimer. However, it must be noted that none of these results preclude the possibility that additional thymine photoproducts are formed on irradiation of DNA and do not survive the conditions of acid hydrolysis.[26] This remains an unsolved problem pending the development of a milder degradation of DNA.[27]

Rearrangement Mechanism. The mechanism of rearrangement of thymine ice–dimer **1** into its isomer **7** can be studied by investigating the degree of incorporation of oxygen-18 into the product when the rearrangement is effected in water enriched with this isotope. If the formation of the disodium salt or any subsequent step involves nucleophilic attack of a hydroxide anion on one of the thymine dimer carbonyls, then half the carbonyl oxygen in the product would be derived from the solvent.

Treatment of thymine dimer with a solution of sodium deuterioxide having an ^{18}O content of 22% gave the corresponding disodium salt of which a part was reconverted into dimer by acidification. This dimer was crystallized from water to remove exchangeable deuterium and then examined by mass spectrometry. Since the mass spectrum was the same as that of a normal sample of thymine dimer and showed no ^{18}O-isotopic enrichment, it must be concluded that the disodium salt is not a bisureido acid **2**, as previously suggested,[1] but is correctly represented as a dihydrate of the diimidate salt **3**.

The rearrangement product **7** was obtained by the addition of bromine to a suspension of the remainder of the disodium salt **2** in oxygen-18-enriched medium and then crystallized from water to remove exchangeable deuterium. Pyrolysis of this material afforded α,α'-dimethylmaleimide (**8**) and 2-imidazolone (**9**) which were purified by sublimation and then examined by mass spectrometry. In both cases, the spectra were identical with those of synthetic samples, showing no change in intensity at (M + 2) or at (M + 4). The possibility that oxygen-18 was incorporated into the rearrangement product but lost by exchange during recrystallization can be eliminated by the known pattern of such behavior in amides.[28–31] These results therefore exclude hydrolytic cleavage at three of the four carbonyl centers of thymine dimer during the formation of **7** and exchange at the fourth, the carbonamido group in **7**, appears unlikely to be associated with C–N bond cleavage. These results are in accord with the following mechanism.

The formation of 3,3′-dibromothymine dimer **4** is analogous to the formation of 3,3′-dimethylthymine dimer from the disodium salt with methyl sulfate.[32] Hydroxide-catalyzed ring fission of the dibromide **4**, which can now ionize only at N-1, produces an isocyanate with expulsion of a bromamide anion **5**. Attack by this anion on the neighboring 4′-carbonyl and addition of the isocyanate to the adjacent 1′-amide nitrogen pro-

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vide the 5.4.5 ring system **6**. The former step presumably is preferred over the alternative loss of bromide and rearrangement to give a second isocyanate function; the latter step is commonly observed in the formation of alkylacylureas as by-products in the Hofmann rearrangement.[33] Finally, debromination of **6** on warming in aqueous acid to give the rearrangement product **7** is a step with long-established precedent.[34,35]

This scheme satisfactorily accounts for the lack of oxygen-18 incorporation into the final product during the above reaction sequence. Although no closely analogous process is known, a similar ring cleavage leading to the formation of an isocyanate has been proposed to account for the rearrangement of certain cytosine derivatives.[36]

It is clear from the proposed mechanism that the formation of *cis,cis,cis*-3-carbonamido-1,7-dimethyl-3,5,9-triazatricyclo[5.3.0.0^{2,6}]deca-4,8,10-trione (**7**) is possible only as a result of *cis–syn* stereochemistry of thymine dimer.

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The Environment of a Reporter Group at the Active Site of Chymotrypsin[1]

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Abstract: The reaction of 2-bromoacetamido-4-nitrophenol with chymotrypsin produces a reporter-labeled protein in which the phenolic group is covalently attached to the methionine residue, Met_{192}, three residues away from the active serine. The modified enzyme is still active but the reporter group apparently is bound at or near the binding site of the enzyme and decreases the velocity of acetyltyrosine ethyl ester hydrolysis. The pH dependence of the absorption spectra indicates that a positively charged group of $pK = 7$ is spatially close to the hydroxyl group of the phenol and that the positively charged group perturbs the pK of the phenol strongly. Other groups of pK's greater than 9 affect the spectra appreciably but less strongly. Binding of substrate or reaction with phenylmethanesulfonyl fluoride eliminates the $pK = 7$ effect apparently by displacing the reporter group from its nearness to the $pK = 7$ group. The $pK = 7$ group is tentatively identified as the histidine at the active site. The results illustrate the value of such environmentally sensitive groups for exploring the microscopic environment of parts of the active site.

The advent of crystallography and the improved methods for protein modification have made the study of the mechanism of enzyme action both more rewarding and more precise. As these studies unfold, it is imperative that the nature of the active site be probed with increasingly sophisticated techniques to discover those environmental influences which make enzymes so effective. Although many physical properties of the protein can be followed as a function of substrate binding, *e.g.*, the over-all hydrodynamic properties, the absorption spectra, optical rotatory dispersion, etc.,[2] these methods suffer from the disadvantage that they represent the integrated changes of a number of different groups. Thus, the change in one group toward a more hydrophobic environment might be nullified by the change in another group toward a more hydrophilic environment. By placing a sensitive "reporter group" at one specific position in a protein molecule the changes in its local environment can be examined. Thus, it was shown that the attachment of an environmentally sensitive nitrophenol moiety near the active site of chymotrypsin produced absorption spectra of the reporter group that were sensitive to substrate binding and that varied with the individual substrates absorbed.[3] In the present paper the environment of this reporter group is probed further both in relation to its immediate neighbors at the active site and in relation to the influence of bound substrate molecules.

(1) The authors wish to acknowledge the generous support of the National Science Foundation and the National Institutes of Health during the course of this work. Abbreviations: Rp, reporter group; RpBr, 2-bromoacetamido-4-nitrophenol; RpH, 2-acetamido-4-nitrophenol; ChTr-Met_{192}Rp, chymotrypsin with above reporter group attached to Met_{192} through a sulfonium linkage; ChTr-Met_{192}Rp-Ser_{195}-PMS, chymotrypsin with reporter group on Met_{192} and phenylmethanesulfonyl group on Ser_{195}; ATEE, acetyltyrosine ethyl ester; DIP, diisopropylphosphoryl; PMSF, phenylmethanesulfonyl fluoride. An account of this work was presented at the 152nd National Meeting of the American Chemical Society, Miami Beach, Fla., April 1966.

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Experimental Section

Synthesis of 2-Bromoacetamido-4-nitrophenol (RpBr). The procedure of Newbery and Phillips[4] for treatment with acetyl halides was followed in general. Crystals of the product, which formed on the dropwise addition of 9.3 ml (0.11 mole) of bromoacetyl

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