Sequence Determination in the Deoxyribonucleotide Series by Stepwise Chemical Degradation[1]

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Abstract: Studies toward a method for the determination of base sequence in deoxyribooligonucleotides are described. Model compounds are oxidized at the 3′-hydroxyl end, terminal nucleobase and the next lower nucleotide with 3′-terminal phosphate are liberated, the latter is removed with phosphomonoesterase, and the cycle is repeated. As currently constituted, interference caused by depurination is suppressed and efficacy with increasing chain length is demonstrated. Shortcomings of the method include rapidly decaying signal strength.

Considerable progress is being made in the elucidation of the primary structure of RNA molecules.[2] Thus, the nucleotide sequences of several homogeneous tRNA's have been completely assigned.[3] On the other hand, corresponding attempts with deoxyribonucleic acids have not fared nearly as well. Aside from the formidable problems of homogeneity, double strandedness, circularity, and sheer size of the DNA molecule, the absence of the *cis*-1,2-glycol system in the sugar moiety as well as the lack of specificity of nuclease cleavage procedures have seriously hampered efforts in this field. In the present paper we report experiments aimed toward sequence mapping by stepwise chemical degradation.[4]

Any such degradative approach must proceed with fixed polarity; *i.e.*, a sequence must be unravelled from either the 3′ or the 5′ end of the chain. Prior attempts have sought to attain this objective by chemical reagents which discriminate between the primary (5′) and the secondary (3′) hydroxyl group. Selective oxidation of the former with platinum catalysts and either molecular oxygen[5] or hydrogen peroxide[6] results in the formation of a uronic acid. The latter was envisaged to be susceptible to degradation, and although the desired β elimination of "nor" nucleotide was not an efficient process in the case of the acid itself,[5] prior conversion to an amide[6] made such an elimination, with concomitant liberation of the 5′-proximate nucleobase (the "signal"), a practical possibility. Some oxidation of the secondary hydroxyl, however, was also observed, threatening simultaneous liberation of the 3′-proximate base[7] (the "noise"), and thus scrambling the polarity of the process.

The problem seemed ripe for further study, however, because of two developments. Recently, mild and efficient oxidation procedures have become available (*vide infra*) which appeared to be of special value in the carbohydrate field. Furthermore, effective discrimination between the 5′ and 3′ end can now, in principle, be furnished by enzyme specificity. Thus, the discovery of 5′-hydroxyl polynucleotide kinase,[8] an enzyme which specifically phosphorylates the 5′ terminus of polynucleotides, would permit the preparation of substrate oxidizable at the 3′ end only. Alternatively, an enzyme has been described which is capable of selective removal of 3′-terminal phosphate[9] (3′-deoxynucleotidase) from polynucleotides.

Figure 1 outlines a scheme in which such enzymes might be employed to impose polarity on the process. Degradation of a trinucleotide serves to illustrate the concept: oxidation of the free 3′-hydroxyl group results in a labile terminus where both nucleobase Z and the next lower ("nor") dinucleoside triphosphate are generated by β elimination. The selective removal of the newly generated 3′-phosphate would then furnish substrate for a second degradative cycle.

The present paper describes experiments with model compounds designed to investigate the feasibility of the oxidative degradation. An alternative scheme utilizing the opposite (5′ → 3′) polarity is under investigation.

Experimental Section

Reagents. Pyridine and dimethyl sulfoxide were distilled and stored over Linde Molecular Sieve Type 4A. Acetic anhydride was purified by distillation. Cation exchanger BioRad AG 50W-X8 was washed with 1 *N* hydrochloric acid and distilled water prior to use. DEAE-cellulose (DE-23, H. Reeve Angel & Co.) was prepared as recommended by the manufacturer. Bacterial alkaline phosphatase (BAP-C) was purchased from Worthington Biochemical Corp.

Paper Chromatography. The following systems were utilized: system A, acetonitrile–0.1 *N* ammonium acetate, pH 7.5 (8:2); system B, acetonitrile–0.1 *N* ammonium acetate, pH 7.5–concentrated ammonia (7:2:1); system C, isopropyl alcohol–concentrated ammonia–water (7:1:2); system D, *n*-butyl alcohol–ethanol–water (50:15:30). Spots were located by scanning with a short-wave ultraviolet lamp (UVS-11, Ultraviolet Products), and cut out for extraction with appropriate buffer. Control paper areas were also excised and extracted. Spectra were measured on a Zeiss PMQ-II ultraviolet spectrophotometer.

(1) Part of this work has been presented at the XXI International Congress of Pure and Applied Chemistry, Sept 4–10, 1967, Prague, Czechoslovakia; see Abstracts of Papers, N-51.

(2) See, for instance, G. G. Brownlee and F. Sanger, *J. Mol. Biol.*, **23**, 337 (1967).

(3) *Inter al.:* R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick, and A. Zamir, *Science*, **147**, 1462 (1965); H. G. Zachau, D. Dütting, and H. Feldmann, *Angew. Chem.*, **78**, 392 (1966); *Z. Physiol. Chem.*, **347**, 212 (1966); U. L. Rajbhandary, S. H. Chang, A. Stuart, R. D. Faulkner, R. M. Hoskinson, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **57**, 751 (1967); B. F. Forger and S. M. Weissman, *Science*, **158**, 1695 (1967).

(4) Several publications have recently appeared utilizing this method in the structure elucidation of certain RNA enzymatic fragments; see H. L. Weith and P. T. Gilham, *J. Am. Chem. Soc.*, **89**, 5473 (1967), and J. X. Khym and M. Uziel, *Biochemistry*, 7, 422 (1968).

(5) G. P. Moss, C. B. Reese, K. Schofield, R. Shapiro, and Lord Todd, *J. Chem. Soc.*, 1149 (1963).

(6) J. P. Vizsolyi and G. M. Tener, *Chem. Ind.* (London), 263 (1962).

(7) G. M. Tener, personal communication as quoted in A. F. Cook and J. G. Moffatt, *J. Am. Chem. Soc.*, **89**, 2697 (1967).

(8) A. Novogrodsky and J. Hurwitz, *Federation Proc.*, **24**, 602 (1965); C. C. Richardson, *Proc. Natl. Acad. Sci. U. S.*, **54**, 158 (1965); A. Novogrodsky, M. Tal, A. Traub, and J. Hurwitz, *J. Biol. Chem.*, **241**, 2933 (1966).

(9) A. Becker and J. Hurwitz, *ibid.*, **242**, 936 (1967).

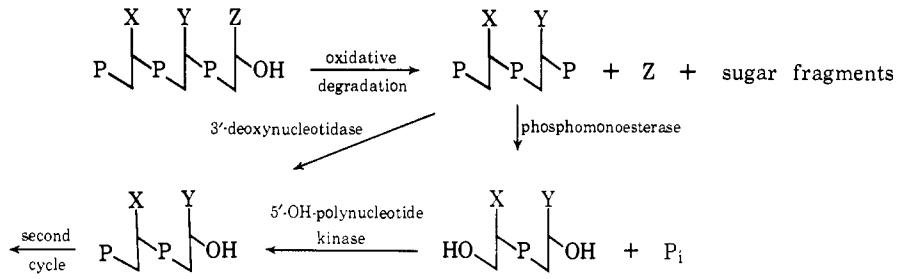


Figure 1. Scheme to impose polarity on the degradative process.

Model Oligonucleotides. The following deoxyribooligonucleotides were prepared analogously to published procedures:[10] oligothymidylic acids,[11] d-pA_{OAc}^{NBz},[12] d-*i*-PrpTpTpT,[13] d-Cl_3CCH_2OpCpT,[14] d-Cl_3CCH_2OpTpA[14] and its diacetate, and d-Cl_3CCH_2OpTpApG.[14]

Nucleotide Oxidations. Oxidations according to Pfitzner and Moffatt[15] as well as Albright and Goldman[16] were carried out as detailed with the pertinent tables and charts. As an example of the routine procedure currently used, the following oxidative degradation of the protected trinucleotide d-Cl_3CCH_2OpTpApG is given in detail.

The trinucleotide (6.0 μmol of triethylammonium salt) was dried thoroughly by lyophilization and taken up in 0.17 ml of dry pyridine. Dry dimethyl sulfoxide (0.3 ml) and 0.2 ml of acetic anhydride were added, and the solution was stoppered under dry nitrogen. The reaction was allowed to proceed at room temperature for 22 hr, 1 ml of methanol was added, and the solution was concentrated under high vacuum to a syrup. The latter was dissolved in water (1 ml); the pH was adjusted to 8.8 by the addition of 0.2 ml of 1.5 *N* triethylammonium bicarbonate (pH 8) plus 0.1 ml of triethylamine base. Bacterial alkaline phosphatase (0.1 mg in 0.01 ml of ammonium sulfate buffer) was added, and the resulting solution was incubated at 37° for 4 hr. It was then transferred to a column (2 × 15 cm) of DEAE-cellulose and elution instituted with a linear gradient of triethylammonium bicarbonate, pH 7.5. (The mixing vessel contained 1 l. of distilled water and the reservoir, 1 l. of 0.3 *M* buffer.) Fractions of 15 ml were collected at the rate of 3 ml per minute, and the ultraviolet absorption at 257 mμ was continuously monitored. An initial sharp peak is due to a pyridine-derived artifact.[1] Nucleobase and derivatives appear in fractions 5–23: these were combined and concentrated to dryness, dissolved in 2 ml of concentrated ammonia, and heated to1 00° in a sealed tube for 1 hr. Subsequent concentration and examination in paper-chromatographic system B indicated the presence of guanine only. This was confirmed by an ultraviolet spectrum. The yields of this and subsequent steps are compiled in Table V.

The nucleotidic material emerged in several peaks later in the chromatogram, the heterogeneity being due to acylation of starting material—making the latter resistant to degradation in the next round—as well as product. All of this ultraviolet-absorbing material was combined, concentrated to dryness repeatedly in order to remove all of the volatile buffer, and a second cycle undertaken. Work-up as above gave a base (adenine) and a nucleotide fraction.

A final oxidative cycle gave the thymine signal, which, however, required a further step in the separation scheme: thymine is the only common nucleobase not separated from the initial ultraviolet peak emerging from the column. Hence, fractions containing this peak were combined, concentrated to dryness, and examined for the presence of nucleobases by paper chromatography in system A, which is capable of resolving the artifact from the nucleobases. The presence of thymine could be clearly noted. The entire fraction was then passed over a small column (0.9 × 12 cm) of BioRad AG 50W-X8 (hydrogen form) and eluted with distilled water. Only thymine emerged; the artifact was retained on the resin and the quantity of the former was determined from its ultraviolet spectrum.

Results and Discussion

In a stepwise sequence study of a DNA fragment, it is necessary to operate on one of its two ends in such a manner that the main body of the fragment remains unaffected, at least for the purpose of the analysis itself. Specific oxidation of a free alcoholic hydroxyl group appears to be a reasonable approach, since the subsequent desired β elimination of nucleobase and phosphate end affects only positions properly located with respect to the site of oxidation, *i.e.*, terminal positions. Of the many methods of oxidation known to organic chemistry, only those which function in a medium where nucleic acid fragments can be solubilized, and which allow maximum specificity and yield as well as minimum interference with the analytical readout and subsequent manipulation, need be considered.

Several oxidation procedures of the nucleoside 3′-hydroxyl group have been reported. The use of manganese dioxide,[17] chromium trioxide in pyridine,[18] platinum, and oxygen under forcing conditions[19] and dimethyl sulfoxide (DMSO) in conjunction with condensing agents[20] have been described. These latter—the Pfitzner–Moffatt oxidation and related procedures—have proved especially useful because of their mildness and efficiency. Thus, although nucleoside derivatives can be converted preparatively to their 2′- or 3′-keto congeners,[21] monodeoxyribonucleotides are, under the conditions of the original Pfitzner–Moffatt oxidation, degraded further into inorganic metaphosphates, sugar fragments, and nucleobase.[7] Use of the sulfoxide–carbodiimide reaction in the stepwise degradation of deoxyribooligonucleotides was proposed in the original communication of Pfitzner and Moffatt,[22] the concept being illustrated in Chart I.

Our own work along these lines indicated that the original Pfitzner–Moffatt conditions would have to be modified, since the efficiency of the procedure greatly declined with increasing chain length of oligodeoxy-

(10) For nomenclature, see IUPAC–IUB Combined Commission of Biochemical Nomenclature: Revised Tentative Rules, as in *Arch. Biochem. Biophys.*, **115**, 1 (1966).

(11) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

(12) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).

(13) Prepared analogously to the corresponding methyl ester: H. G. Khorana, *ibid.*, **81**, 4657 (1959).

(14) These compounds were prepared by the general procedures of F. Eckstein, *Chem. Ber.*, 100, 2236 (1967). Details are to be published at a later date.

(15) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 5661 (1965).

(16) J. F. Albright and L. Goldman, *ibid.*, **87**, 4214 (1965).

(17) A. S. Jones, R. T. Walker, and A. R. Williamson, *J. Chem. Soc.*, 6033 (1963).

(18) A. S. Jones, A. R. Williamson, and M. Winkley, *Carbohydrate Res.*, **1**, 187 (1965).

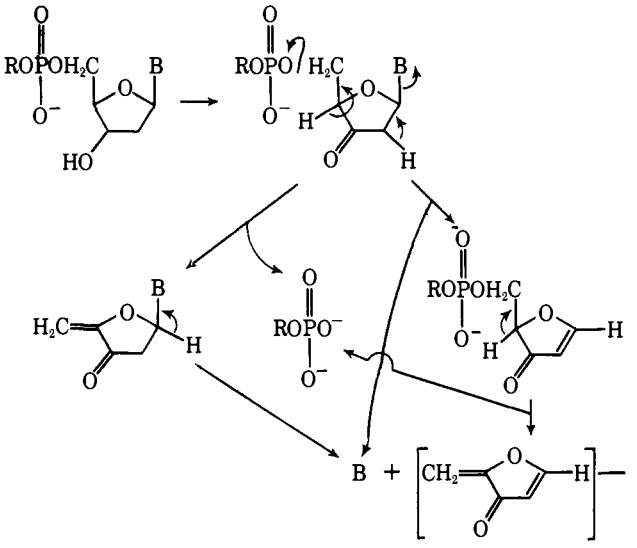
(19) See ref 7.

(20) Reviewed by N. Kharasch and B. S. Thyagarejan, *Quart. Rept. Sulfur Chem.*, **1**, 5 (1966). See especially pp 16, 52 ff, also W. W. Epstein and F. W. Sweat, *Chem. Rev.*, **67**, 247 (1967).

(21) See Cook and Moffatt, ref 7.

(22) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, **85**, 3027 (1963).

Chart I. Oxidative Degradation



B = nucleobase
R = nonterminal oligonucleotide

ribonucleotide substrate (Table I). Thus, while the mononucleotide gave rise to a very strong "signal," no nucleobase could be detected after attempted degradation of a model trinucleotide.

Table I. Oxidation by the Method of Pfitzner and Moffatt[15]

	Substrate		
	pT	pTpT	*i*-PrpTpTpC
Yield of nucleobase, %	116[a]	46	Not detectable

[a] This artificially high figure, already noted by Pfitzner and Moffatt in their original communication,[22] is no doubt due to contaminating pyridine derivative. The mononucleotide was oxidized according to the literature.[15] The oligonucleotides, 10–15 μmol, of thoroughly dry material, were dissolved in 3–4 ml of dimethyl sulfoxide and treated with 2 mmol of dicyclohexylcarbodiimide (DCC) and 0.1 mmol of pyridinium acetate at room temperature for 18 hr. Water (3 ml) was added, and the suspension was extracted with ether and filtered. The aqueous portion, after concentration to 2 ml (pH 8.5), was partitioned on a 2 × 15 cm column of DEAE-cellulose (bicarbonate cycle). Elution with water gave the nucleobase fraction, which was concentrated and further characterized and quantized by paper chromatography in systems A and B. The remaining nucleotide fraction was eluted with 1.5 *M* triethylammonium bicarbonate buffer, pH 7.5, and measured by ultraviolet absorption.

Accordingly, we applied the modification of Albright and Goldman[16] to nucleotide oxidations, and found it to be an effective method for this purpose. Figure 2 illustrates its application to a deoxyribotrinucleotide; the 5′-isopropyl ester of d-pTpTpC was subjected to oxidation with dimethyl sulfoxide–acetic anhydride for 18 hr at 70°. The product was partitioned into nucleobase and a phosphorus-containing fraction by passage over a column of DEAE-cellulose. The nucleobase fraction, eluted with distilled water, was hydrolyzed and quantitatively determined by paper chromatography or tlc. The nucleotide fraction, eluted with 1.5 *M* triethylammonium bicarbonate, was treated with bacterial alkaline phosphatase and subjected to a second oxidative cycle. At this point, some 5% of the nucleobase signal was again C, in addition to the expected,

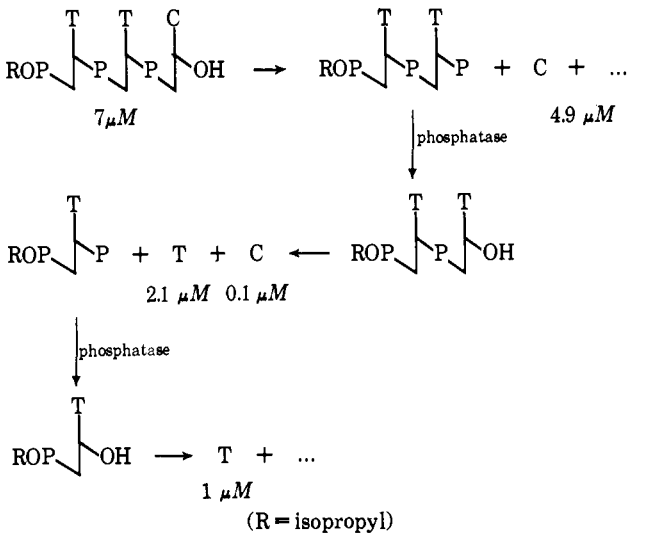


Figure 2. Pyrimidine trinucleotide degradation by the method of Albright and Goldman.[16] The indicated quantity of oligonucleotide was dissolved in 0.3 ml of DMSO and 0.2 ml of acetic anhydride under nitrogen, and the solution was heated for 18 hr at 70°. All volatiles were removed *in vacuo;* the residue was taken up in 2 ml of water, pH adjusted to 6.8 with triethylamine, and passed over a column of DEAE-cellulose, 2 × 15 cm. The base fraction was eluted with water and analyzed and characterized as usual. The phosphorus-containing fraction was eluted with 1.5 *M* triethylammonium bicarbonate, pH 7.5, concentrated to dryness with concomitant removal of the volatile buffer, and dephosphorylated with bacterial alkaline phosphatase. The cycle was then repeated, indicated quantities of base being measured.

and predominant, T. The origin of this "noise" is ascribed to that portion of the trinucleotide which survived the first cycle. In more recent modifications (*vide infra*) of the procedure, even the moderate level of "noise" has been apparently eliminated.

The difficulty with the Albright–Goldman method, however, was that it did too much: with purine oligonucleotides, depurination was observed. The factors responsible for this event were studied as shown in Table II; a blocked purine nucleotide was subjected to the conditions shown, and its survival measured.

It can be seen that depurination occurred not only in the presence of acetic anhydride (expt 1, 3, and 4), but even in neat DMSO (expt 2), and that lower temperatures do not eliminate this difficulty (expt 4). It was possible to avoid this complication, however, by the addition of pyridine (expt 5 and 6). The point is also illustrated in the following experiment (Figure 3): a properly blocked purine-containing deoxyribodinucleotide gave rise to signal only in the presence of 3′-hydroxyl; that is, degradation must be initiated by oxidation, as desired.

Some other modifications of the oxidation conditions are shown in Table III; it can be seen that pT can be degraded to an extent approaching 80%. The conditions which give this result are defined in expt 6.

What is the effect of increasing chain length on the yield of nucleobase signal? A series of oxidations were carried out on polythymidylic acids; the results are summarized in Table IV. It can be seen that while there may be a small initial depression of the yield of recovered thymine, the apparent trend is certainly reversed with the hexanucleotide, and good signal strength is maintained up to the largest oligomer studied. The

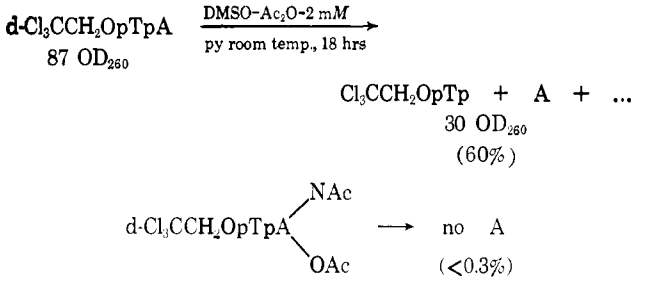
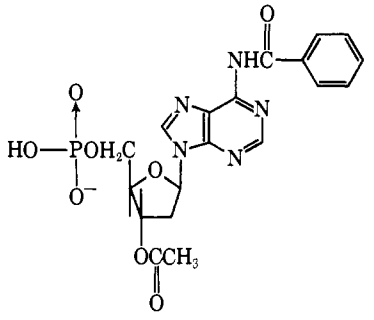


Figure 3. Depurination. Procedure as in experiment 5, Table II, except that the nucleobase fraction (water eluate from the DEAE-cellulose column) was quantitatively analyzed for adenine by paper chromatography in system B.

nature of the counterion seems to have some effect: while good yields of nucleobase are obtained from the free acid, pyridine, ammonium, and substituted ammonium salts, salts of strong bases, *e.g.*, sodium, perform very poorly.

Table II. Depurination[a]



Expt	Reagents	Temp, °C	Nucleotide recd, %
1	0.3 ml of DMSO 2 m*M* Ac_2O	70	11
2	0.3 ml of DMSO	70	59.5
3	0.2 ml of Ac_2O	70	18.7
4	0.3 ml of DMSO 2 m*M* Ac_2O	23	69
5	0.3 ml of DMSO 2 m*M* Ac_2O 2 m*M* py	70	96
6	0.3 ml of DMSO 2 m*M* Ac_2O 2 m*M* py	23	111

[a] Approximately 100 OD_{303} units of blocked nucleotide were oxidized for 18 hr under the conditions shown, and the reaction mixture was quenched with water. Passage over a DEAE-cellulose (bicarbonate cycle) column and elution with water was followed by 1.5 *M* triethylammonium bicarbonate buffer, pH 7.5. The uv spectrum of the buffer eluate was used to calculate the percentage of recovered nucleotide.

Application of these conditions to the individual 5′-deoxyribonucleotides is shown in Figure 4. The release of nucleobase (in per cent of theory) is plotted against time. There are certain differences in behavior to be noted: the initial rate of oxidation is faster for d-pC and d-pG on the one hand than for pT and d-pA on the other; the eventual yields are somewhat higher (~80%) in case of the latter (pT and d-pA), however, as compared to the former (~60%). Furthermore, a definite decrease in yield is noted with prolonged oxidation periods in the case of d-pA, and, to a lesser extent, with

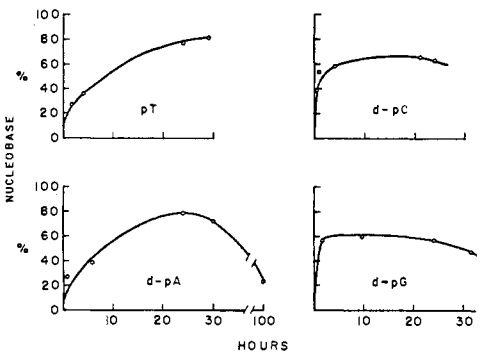


Figure 4. Oxidation of 5′-deoxyribonucleotides. Approximately 100 OD_{260} (pH 7) of nucleotide was thoroughly dried and dissolved in 0.30 ml of dimethyl sulfoxide. Pyridine (0.17 ml) and 0.20 ml of acetic anhydride were added, and the reaction vessel was flushed and stoppered under dry nitrogen. Aliquots containing 1.5 OD_{260} units were removed at appropriate intervals and spotted on Whatman filter paper 3MM. Paper chromatography in system B (for d-pC and d-pG oxidations), system C (d-pA), and system D (pT) resolved the nucleobases generated (or their derivatives). Extraction of the appropriate regions and measurement of their ultraviolet absorption spectra generated the data displayed in the graphs.

d-pG. A compromise, taking these various factors into account, leads to the reaction period of 22 hr adopted for routine procedure.

Table III. Oxidative Degradation without Depurination: Efficacy[a]

Expt	Reagents	Temp, °C	Nucleotide surviving, %
1	2 m*M* DCC	23	0
2	0.2 m*M* Ac_2O 0.2 m*M* py	23	31
3	2 m*M* Ac_2O 2 m*M* py	23	28.5
4	0.3 ml of dimethylacetamide 2 m*M* Ac_2O 0.4 m*M* triethylamine	23	25
5	2 m*M* Ac_2O 2 m*M* py	70	19
6	0.3 ml of dimethylacetamide 2 m*M* Ac_2O 2 m*M* py	23	11

[a] 10 μM of pT in 0.3 ml of DMSO were treated as shown for an 18-hr period. Subsequent procedure as in Table II.

Table IV. Oxidative Degradation of Chemically Synthesized Thymidylic Acids[a]

Oligomer	Yield of thymine, %	Oligomer	Yield of thymine, %
pT	89	pT_6	75
pT_3	68	pT_7	82
pT_4	74	pT_8	101
pT_5	65	pT_9	100

[a] Approximately 100 OD_{260} units were treated as described in the legend to Table III (expt 5). The crude oxidation product was passed over Dowex 50-X8 (hydrogen) to remove the pyridine-derived artifact, and thymine was separated from surviving nucleotidic material by DEAE-cellulose chromatography (see Experimental Section). Total recoveries were essentially quantitative. Hyperchromicity was not taken into account but is not expected to affect figures substantially: the experimentally (venom diesterase digestion) determined hyperchromicity of pT_7 was 5.45%.

Table V. Base Sequence of the Model Trinucleotide d-Cl_3CCH_2OpTpApG (6.0 μM)

Step	Yield from oxidative degradation[a] G	A	T
1	3.5	<0.1	<0.1
2	<0.1	2.0	<0.1
3	<0.1	<0.1	1.9

[a] In μmoles of nucleobase.

Table VI. Base Sequence Determination of the Model Dinucleotide d-Cl_3CCH_2OpCpT (10.4 μmol)

Step	Yield from oxidative degradation[a] C	T
1	4.9	<0.15
2	<0.10	<3.0

[a] In μmoles of nucleobase.

Application of the method to a model containing purine bases could now be undertaken. An appropriate model, d-Cl_3CCH_2OpTpApG, synthesized by the procedures of Khorana as modified by Eckstein,[14] was subjected to three cycles of the oxidation–degradation–monophosphorolysis procedures. The results are displayed in Table V. It will be seen that there is never any question of recognizing the proper base signal: any surviving nucleotide is largely protected from subsequent degradation—at the wrong time—presumably by acetylation. The decay of signal strength appears to be greatest at step 1; this seems to be because of the inherent sluggishness of G. Guanosine nucleotides give rise to several products directly upon oxidation; subsequent ammonia hydrolysis to free nucleobase appears to entail some loss. This does not, however, lead to signal ambiguity since no confusing ultraviolet paper spots or spectra were discerned.

Finally, an additional model possessing an internal cytosine was subjected to the analytical procedure since, like d-pG, the yield of nucleobase from its nucleotide reaches a lower yield figure than in the case of the adenine and thymine deoxyribonucleotides. As can be seen from Table VI, unequivocal results were obtained in this case as well.

In summary, it may be stated that the method here described is capable of determining the sequence of model deoxyribooligonucleotides. The limits of its applicability are under study.

Acknowledgment. Thanks are due to Messrs. O. Keller and E. Heimer for the preparation of certain model deoxyribooligonucleotides, and to Dr. C. Harvey for his help with enzymatic procedures.

Biosynthesis of Capsaicin and Dihydrocapsaicin in *Capsicum frutescens*[1]

Edward Leete and Mary C. L. Louden

Contribution from the School of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received May 29, 1968*

Abstract: Capsaicin (N-(4-hydroxy-3-methoxybenzyl)non-*trans*-6-enamide) and 6,7-dihydrocapsaicin are found in approximately equal amounts in *Capsicum frutescens*. These alkaloids, isolated from the fruits of the plant 2 weeks after the administration of DL-phenyl-3-^{14}C-alanine, were labeled solely on the methylene group of their vanillylamine residues. The administration of DL-tyrosine-3-^{14}C afforded alkaloids with very low activity. L-Methionine-methyl-^{14}C afforded radioactive alkaloids, and degradation of the dihydrocapsaicin indicated that all the activity was located on the methoxy group. No activity was found in the alkaloids when DL-mevalonic-2-^{14}C acid or DL-leucine-1-^{14}C were fed to the plant. However, radioactive capsaicin resulted when L-valine-U-^{14}C was fed. Essentially all the activity was located at C-7, -8, -9, and -10 of the 8-methyl-6-nonenic acid moiety of the alkaloid.

Capsaicin (**1**) and its dihydro derivative **2** occur in the fruit of *Capsaicin* species[2] and are responsible for the hot taste of the peppers. In *Capsicum annum* Kosuge and coworkers[3] found capsaicin and its dihydro derivative to be present in the ratio of 7:3. More recently Bennett and Kirby[4] reported the following composition of the phenolic components of *C. annum:* capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%). The presence of the three minor components was established by mass spectrometry.

We commenced work on the biosynthesis of capsaicin in 1965 and have used the plant *C. frutescens* in all our experiments. We found that the phenolic fraction from the fruits of this species contained 47% capsaicin and 53% dihydrocapsaicin. Separation was initially achieved by thin layer chromatography on silica gel plates impregnated with silver nitrate. A more convenient method involved O-methylation, followed by reaction of the mixture with osmium tetroxide. The 6,7-diol **3** derived from capsaicin was readily separated from O-methyldihydrocapsaicin (**4**) by column chroma-

(1) An account of this work was presented at the 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1967. This investigation was supported by a research grant, GB-3696, from the National Science Foundation.

(2) S. Kosuge, Y. Inagaki, and K. Uehara, *Nippon Nogei Kagaku Kaishi*, 32, 578 (1958). These authors assigned the names capsaicin I and II to compounds **1** and **2**, however we prefer the more descriptive names.

(3) S. Kosuge, Y. Inagaki, and H. Okumura, *ibid.*, 35, 923 (1961).

(4) D. J. Bennett and G. W. Kirby, *J. Chem. Soc., C*, 442 (1968).