chim. Biophys. Acta 145, 292.
Mans, R. J., and Novelli, G. D. (1961), *Arch. Biochem. Biophys. 94*, 48.
Muench, K. H., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper and Row, p 375.
Ralph, R. K., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83*, 2926.
Rosenberg, M., and Gilham, P. T. (1971), *Biochim. Biophys. Acta 246*, 337.
Roychoudhury, R. (1972), *J. Biol. Chem. 247*, 3910.
Schott, H. (1972a), *Angew. Chem. 84*, 819.
Schott, H. (1972b), *Angew. Chem., Int. Ed. Engl. 11*, 824.
Waters, L. C., and Novelli, G. D. (1968), *Biochem. Biophys. Res. Commun. 32*, 971.
Weith, H. L., Wiebers, J. L., and Gilham, P. T. (1970), *Biochemistry 9*, 4396.
Yang, W.-K., and Novelli, G. D. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 208.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

Periodate Oxidation and Amine-Catalyzed Elimination of the Terminal Nucleoside from Adenylate or Ribonucleic Acid. Products of Overoxidation†

Mayo Uziel

ABSTRACT: The kinetic constants of the periodate oxidation of compounds derived from the amine-catalyzed elimination of phosphoric esters differ for mononucleotide and RNA substrates. Both substrates show kinetic curves similar to that for glycol oxidation. The reaction is first order in periodate, and 2 mol of formic acid is produced at the same rate as the free base. CO_2 is formed from overoxidation of formate or the three-carbon fragment from the elimination step. Our data do not support Rammler's hypothesized mechanism for elimination and overoxidation. The evidence suggests that ether cleavage (C-4′–O–C-1′) occurs before overoxidation.

Ribonucleic acid can be sequentially degraded by the combined action of periodate and primary amines on terminal nucleotides, which leads to cleavage of the phosphoric ester linkage (Khym and Cohn, 1961; Whitfield and Markham, 1953; Uziel and Khym, 1969). In the presence of excess periodate, the nucleoside fragment is further degraded to free base (Neu and Heppel, 1964). The compound formed immediately upon elimination of the phosphoryl ester has not been identified, although there has been speculation on its structure (Rammler, 1971). As this product is also the substrate for the overoxidation,[1] discussions of elimination and overoxidation are not always mutually exclusive. For example, Khym (1963) has isolated an amine adduct (18, Figure 2) in the elimination reaction, and Rammler (1971) has used this observation to support his mechanism for elimination and overoxidation.

The overoxidation process is defined as the requirement for a large excess of periodate to cleave the remaining carbohydrate material to release the base (Neu and Heppel, 1964; Tankó *et al.*, 1967). The substrate for this reaction is the amine-catalyzed elimination product. The products of the overoxidation include formate and CO_2, an observation which has provided the basis for the description by Rammler (1971) of a novel mechanism for the elimination and overoxidation. The author has examined the chemistry of these reactions with a view toward optimizing the overoxidation and elimination conditions for sequential degradation of RNA. This paper concerns the overoxidation step; the detailed kinetics and mechanism of elimination will be discussed in a separate publication.

Materials and Methods

All reagents were of the highest purity available. $NaIO_4$ was purchased from Matheson Coleman Bell Co., ornithine was obtained from Calbiochem, and the RNA was prepared by phenol extraction from *Escherichia coli* B. Purification of the tRNA fraction was by DEAE chromatography (Nishimura *et al.*, 1967). Carbon dioxide formation was measured by a nitrogen gas transfer of the released gas to a standardized solution of barium hydroxide, followed by titration of the excess alkali with standard HCl. Bases were measured spectrophotometrically after cation-exchange chromatography at pH 5.2 (Uziel *et al.*, 1968; Uziel *et al.*, 1971). Cytidine and other nucleoside derivatives were measured on a 15-cm column of cation-exchange resin according to Table V in Uziel *et al.* (1968). Optical rotations were measured in 2-dm cells in a Rudolph polarimeter at room temperature (23°). Phosphate was measured by the technique of Hurst and Becking (1963).

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[1] Overoxidation is defined as a requirement for excess periodate at elevated temperatures to release the purine or pyrimidine attached to the elimination product. Since we have been unable to isolate an intermediate step in the conversion of the *elimination product* to the *overoxidation substrate*, both these terms are used to refer to the compounds present in solution after elimination, when no excess periodate is present. They are used individually to emphasize the properties of the elimination reaction and the overoxidation reaction, respectively.

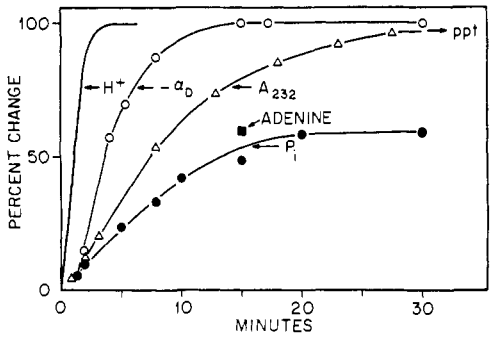

FIGURE 1: Changes occurring during the dephosphorylation and overoxidation of adenylate. Adenylate (0.033 M, final concentration) was treated with an equimolar concentration of periodate in 0.22 M ornithine at pH 7 and 23°. Acid production was measured in a Radiometer TTT-1 stat. The rotation was measured in a 2-dm tube at approximately 1-min intervals with a sodium lamp source. The change in absorbance at 232 nm was measured in split cells, where the reference contained the adenylate and periodate in one compartment and the amine in the second compartment. The rotation and absorbancy changes are reported as percentages of the maximum value at 60 min. For adenine formation, orthophosphate formation, and proton release, 100% is equal to 1 mol/mol of adenylate. To measure released phosphate, the reaction was stopped by addition of 0.5 vol of 1 M sodium borohydride to aliquots from the reaction mixture. The reaction mixture was acidified with 0.5 vol of 1 M acetic acid, and the degassed solution was passed through a column containing about 0.3 ml of charcoal (acid washed) and ~1 ml of Dowex 50 H^+. The column was washed with water to recover all the inorganic phosphate. The pseudo-first-order rate constants (min^{-1}) for each reaction are $k_H = 0.63$, $k_\alpha = 0.29$, $k_{A232} = 0.11$, and $k_P = 0.12$.

Results

Since the substrate(s) for the overoxidation has not been isolated or characterized we prepared them by carrying the elimination to greater than 97% elimination (5 half-times) (Figure 1). We then added excess periodate to begin the reaction. To ensure that no further changes were occurring in the substrate we examined several of the chemical changes accompanying the elimination (Figure 1). The reactions illustrated in Figure 2 summarize the potential chemical pathway(s) consistent with the observations on elimination. (Figure 1 illustrates the measured changes.) Equations 1–3 (Figure 2) are common to all schemes and represent carbinolamine and aldimine formation with concomitant release of acid, since these compounds have pK values considerably less than the parent amine (Hupe *et al.*, 1972). The elimination product(s) (**8**, **13**, or **14**, Figure 2) has one less asymmetric carbon than the parent molecule (**2**, Figure 2); thus there should be rotation changes accompanying elimination. In addition, the unsaturated aldimines (**8**, **13**, and **14**, Figure 2) should show a new spectrum (Steinschneider, 1971), and orthophosphate should be released from the 5′-adenylate substrate. On overoxidation unsubstituted adenine is formed. All these phenomena reach a plateau after 20 min. The only subsequent changes are precipitate formation (contains iodate) and a very slow rotation change toward zero. The incompleteness of the reaction under these conditions has been recorded by others (Steinshneider, 1971; Neu and Heppel, 1964). This side reaction is pH dependent (Uziel, unpublished[2]), and it is temperature dependent and minimal at 45° (Neu and Heppel, 1964).

[2] Uziel, M., unpublished data.

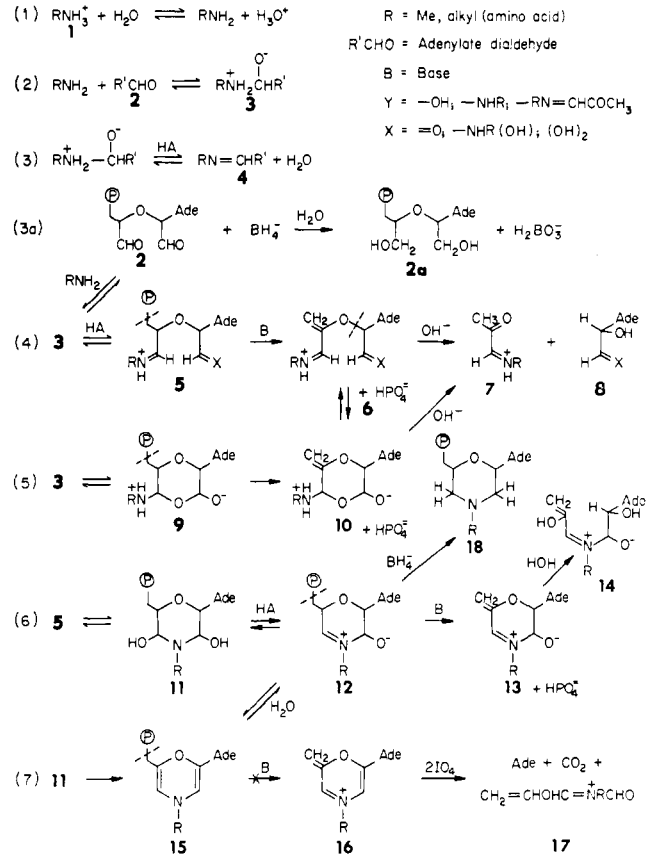

FIGURE 2: Multiple equilibria that may be present in the reaction solution. Steps 1–3 outline the combination of an amine with an aldehyde to form a Schiff base with the elimination of one proton and 1 mol of water. Step 3a illustrates the reduction of the dialdehyde by borohydride. Steps 4, 5, and 6 outline possible pathways for the elimination of phosphate from the 5′ position. As indicated in the figure, both the aminated and hydrated forms of each aldehyde, as well as cyclic forms, have a reasonable probability to exist (Guthrie and Honeyman, 1959). The borohydride reduction of **12** with the intermediate formation of the isomeric Schiff base at C-2′ can lead to the known product **18** (Khym, 1963). Products **9** and **10** may exist in solution but probably do not function in the elimination reaction. Equation 7 outlines Rammler's (1971) hypothesis for overoxidation. Compound **15** is Rammler's suggested intermediate in the elimination process.

Reaction Order. Direct measurement of the loss of periodate was complicated by the changes in the product spectrum during overoxidation and by side reactions that consumed periodate (see Table I). The reaction order was determined by the procedure described by Frost and Pearson (1961), where the ratio $(\log v_2 - \log v_1)/(\log [IO_4^-]_2 - \log [IO_4^-]_1)$ is equal to the reaction order. The calculated reaction order is 1 ± 0.2 (four rate measurements) for both adenylate and RNA as substrate (Figure 3). Thus, only 1 mol of periodate is involved in the rate-determining step.

Concentration Dependence. The mechanism of periodate oxidation of glycols follows saturation-type kinetics, where there is a rapid equilibrium formation of a complex followed by rate-determining breakdown of the complex to the aldehydes plus iodate (Duke, 1947). The shape of the rate *vs.* periodate curve is the same as the simple enzyme *vs.* substrate curve. The overoxidation of the RNA substrate also follows this pattern (Figure 3). The overoxidation rate of the mononucleotides did not reach a plateau at 0.014 M IO_4^-. We did not go to higher periodate concentrations because periodate loses

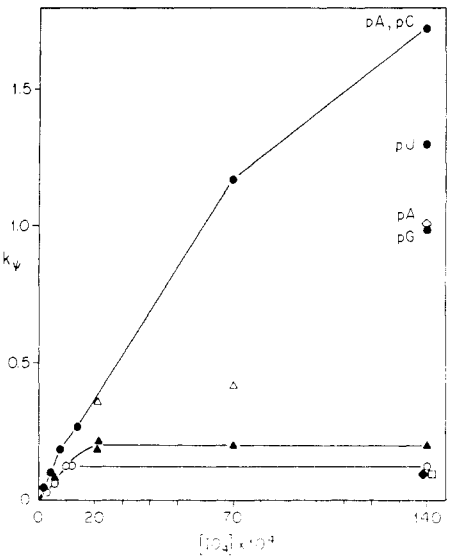

FIGURE 3: Comparison of overoxidation rates when the elimination product was generated in the presence and in the absence of excess periodate. All reactions were run at 0.2 mM substrate and 0.25 M ornithine (pH 6.5) at 47°. To generate the elimination product prior to adding excess periodate, the substrate was initially oxidized with an equimolar amount of periodate (2 min at 47°) in the presence of amine (10 min for pA, 40 min for RNA) to give total (>98%) elimination. A second aliquot of periodate was then added to give the desired final concentration, and the amount of released adenine or cytosine was measured after a suitable interval. The pseudo-first-order rate constants were calculated from the expression $k\psi = 0.693/t_{1/2}$. The half-life was measured graphically from the plot of log [elimination product remaining] *vs.* time. The following substrates for the overoxidation step were treated as described above: (●) pA, pC; pU and pG, single points at 0.014 M IO_4^-; (△) tRNApA and (▲) tRNApC elimination products. The following compounds were treated in the presence of excess periodate from the beginning of the reaction: (◇) pA; (○) tRNA (terminal A or C), ApA, 5′-adenosine methanephosphonate; (◆) ApU; (□) UpCpG. Addition of 2×10^{-4} M phosphate did not change the reaction rates. The RNA was put through one cycle of elimination and overoxidation to remove periodate-sensitive groups on the RNA. After phosphatase treatment cytosine was the end group. The amount of periodate-sensitive material is small, since the original RNA substrate (no pretreatment) gave the same result as the precycled RNA substrate.

TABLE I: Formation of Carbon Dioxide and Adenine during Overoxidation of Adenylate.[a]

Substrate	IO_4^-/S[b]	47° Ade/pA	47° CO_2/S[b]	37° Ade/pA	37° CO_2/S[b]
Adenylate + 0.23 M ornithine	1	0	0[c]	0	0[c]
	2	0.3	<0.05[c]	0.14	0[c]
	4	0.5	0.8	0.14	0.42
	8	1.0		0.28	1.1
Adenylate + 1.5 M methylamine	4		0.23	0.52	<0.07
	4 + acid		0.23		<0.07
0.23 M ornithine + formate[d]	3		0.4		
Gra-3-P[e]	7		0.6		
$NaHCO_3$[f]			0.9		

[a] The oxidation reactions were carried out for at least 20 min in a temperature-controlled, two-vessel, closed system, with nitrogen gas flowing through the reaction vessel and into standardized $Ba(OH)_2$. The excess alkali was titrated with standard HCl, and the moles of CO_2 were calculated by dividing the net alkali loss by 2 (CO_2 is a bivalent acid). The formate and $NaHCO_3$ were 10 mM. CO_2 transfer in the system had a half-life of close to 8 min. All values are given in mole ratios. [b] S is the substrate (all were 0.01 M). [c] Gas transfer, usually 30 min, was extended to 60–80 min. Acidification did not change the results. [d] See Dyer (1956). [e] Gra-3-P is D,L-glyceraldehyde 3-phosphate and was 2 mM. [f] Control experiment for CO_2 recovery.

its oxidative selectivity for glycol at concentrations higher than about 0.04 M, so that compounds such as formate (Table I) are oxidized (Dyer, 1956). The limiting first-order rate constant for oxidation-dependent base formation is 0.21 min^{-1} (k_2 = 1050 M^{-1} min^{-1}) in 0.25 M ornithine for release of cytosine from RNA, and by extrapolation from a plot of $1/k_0$ *vs.* $1/[IO_4^-]$ a value of 2 min^{-1} (k_2 = 10,000 M^{-1} min^{-1}) is obtained for release of adenine or cytosine from their corresponding mononucleotides. These values are in the range observed for a variety of α-glycols (Duke, 1947; Honeyman and Shaw, 1959). The differences in rates are ascribed to conformational differences and the presence of steric bulk (Honeyman and Shaw, 1959). Thus, the different oxidation rates between the mononucleotide and RNA elimination products suggest that the two overoxidation substrates do not have the same structure.

The limiting oxidation rate depends on the time of addition of excess periodate (Figure 3). (One mole of periodate is needed initially to generate the dialdehyde substrate for the elimination.) If the excess periodate is present at the beginning of the reaction (*i.e.*, during dialdehyde formation), then the measured rate is lower (Figure 3) than when the elimination reaction is carried to completion before addition of excess periodate. This is expected if part of the substrate is oxidized to free base before the elimination is complete, thus reducing the substrate concentration for the overoxidation step.

Products of the Reaction. A number of easily identifiable products are expected if the overoxidation mechanism is a simple α-glycol-type cleavage from structures like **24** and **23** in Figure 4. Rammler (1971) has identified formic acid as an early product of the overoxidation of adenylate. We find that 2 mol of acid is released at a rate equal to the rate of adenine formation (Table II). The release of 2 mol of acid is consistent with the proposed pathway (Figure 4). Rammler (1971) has proposed a pathway that involves oxidation of an enamine (**15** → **16** → **17**, Figure 2) based on his observation that CO_2 is released. This path requires an additional 2 mol of periodate and the release of up to 3 mol of acid to completely convert the elimination product to free base. The reaction order (Figure 1) and the acid release data (Table II) are not sufficient to rule out this mechanism, since reaction order specifies only the moles of periodate in the rate-determining step, and the CO_2 is a gas and might be partially lost under the titration conditions. On the other hand, the rate and extent of CO_2 release are not consistent with Rammler's (1971) proposal (Table I). Adenine and CO_2 are not released in quantitatively identical amounts, and the yield of CO_2 is variable. In addition, the overoxidation products are also converted to CO_2 (Table I). It would appear that CO_2 production is primarily a side reaction and not involved in the base-release process.

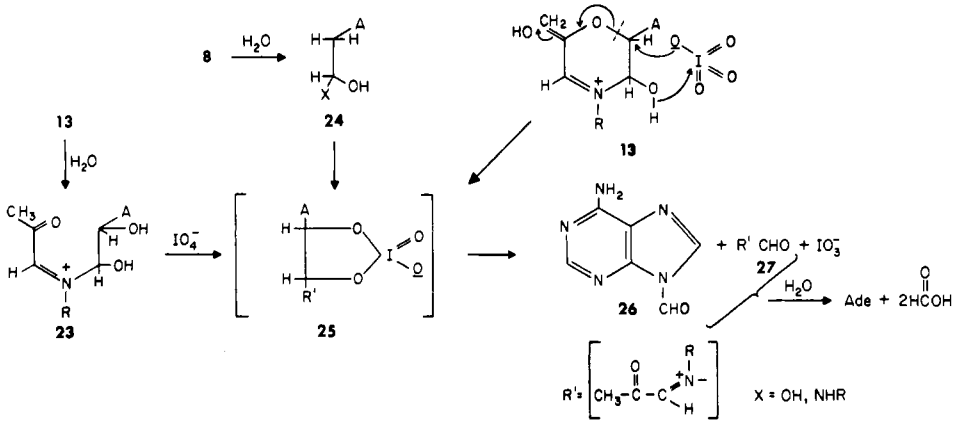

FIGURE 4: Pathway(s) of oxidative release of adenine. Three kinetically equivalent possible pathways are illustrated for two potential substrates (**8**, **13**; see Figure 2). The difference between the paths is in the structure of the reactant combining with periodate. In the paths **8** → **24** (X = NHR or OH) and **13** → **23**, the oxidation mechanism is the same as that for glycol (Duke, 1947). In the path **13** → **25** the ring opening is initiated by periodate, and, as in the other two paths, the slow step is still **25** → **26** to cleave the C-1′–C-2′ linkage. The hydrolysis of the formyl residues on **26** and **27** does not appear to be rate limiting (Figure 3, Table I).

Discussion

The utility of amine-catalyzed elimination of phosphoric esters from periodate-oxidized nucleotides has been recognized for some time, yet no unequivocal picture of the reaction mechanism has been produced. This stems in part from the complexity of the reaction mixture (see Figure 2 for potential intermediates) and partly from the assumption that mononucleotides are good models for the study of the reaction mechanisms (Steinschneider, 1971; Rammler, 1971; Khym, and Cohn, 1961). The demonstration that mononucleotides have substantially different kinetics (Figure 3; Uziel[2]) indicates the oligonucleotide substrates are required for such studies.

The empirical studies of Neu and Heppel (1964) and Tankó *et al.* (1967) show that elimination can be carried to completion at higher temperatures, but the isolatable intermediates are not those predicted by suggested mechanisms (Steinschneider, 1971; Rammler, 1971). We have confirmed Tankó's (1967) observation that the base is removed before the phosphoric ester elimination and that initial cleavage of the C-4′–O–C-1′ ether is the probable path (M. Uziel[2]). A combination of our observations on the overoxidation rate and the observations on the elimination rate for mononucleotide or oligonucleotide substrates (Tankó *et al.*, 1967; Steinschneider, 1971) suggests that the elimination products (overoxidation substrate) are different for the two types of substrate. Three structures that would allow selective periodate oxidation consistent with our data are described in Figure 4.

Transformation of the elimination product to unsubstituted base by periodate overoxidation is useful in sequential degradations. For purposes of analysis, the overoxidation conditions apply to the four usual bases and most of the minor ones. Acid hydrolysis is less reliable because the aldehyde groups are still reactive and the pyrimidine derivatives are hydrolyzed at a considerably slower rate. If the separation process for isolating the elimination product carries even a small amount of the RNA substrate, the oxidation route will oxidize only the elimination product. Hydrolysis techniques are not so selective.

TABLE II: Acid Production during Overoxidation at 47°.[a]

pH	Addition[b]	H^+ Released (mol)	K_ψ (min^{-1})
6.5	pA + IO_4^- (1:1)	1.07 ± 0.05	2.8
6.5	$pA_{(CHNR)_2}$[c] + IO_4^- (1:20)	2.1 ± 0.1	2
	Total	3.17 ± 0.15	

[a] No acid is formed on periodate oxidation of adenylic acid. In the presence of an amine, acid is released due to formation of an amine adduct with a significantly lower pK. [b] The two rows represent two steps in the same reaction mixture in the presence of 0.25 M ornithine. The IO_4^- concentration was raised by the addition of 0.25 M $NaIO_4$. The final concentrations of reagents were $pA_{(CHNR)_2}$, 1 mM, and IO_4^-, 20 mM. The acid release with excess periodate occurs at approximately the same rate as the release of adenine under these conditions (Figure 3). [c] This is the carbinolamine of adenylic acid dialdehyde and ornithine.

Acknowledgments

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References

Duke, J. (1947), *J. Amer. Chem. Soc. 69*, 3054.
Dyer, J. R. (1956), *Methods Biochem. Anal. 3*, 111.
Frost, A., and Pearson, A. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, p 45.
Guthrie, R. D., and Honeyman, J. (1959), *J. Chem. Soc. (London)*, 2441.
Honeyman, J., and Shaw, C. J. G. (1959), *J. Chem. Soc. (London)*, 2454.
Hupe, D. J., Kendall, M. R. C., and Spencer, T. A. (1972), *J. Amer. Chem. Soc. 94*, 1254.
Hurst, R. O., and Becking, G. C. (1963), *Can. J. Biochem. Physiol. 41*, 469.
Khym, J. X. (1963), *Biochemistry 2*, 344.
Khym, J. X., and Cohn, W. E. (1961), *J. Biol. Chem. 236*, PC9.

Neu, H., and Heppel, L. (1964), *J. Biol. Chem. 239*, 2927.
Nishimura, S., Harada, F., Maruskima, U., and Seno, T. (1967), *Biochim. Biophys. Acta 142*, 133.
Rammler, D. H. (1971), *Biochemistry 10*, 4699.
Steinschneider, A. (1971), *Biochemistry 10*, 173.
Tankó, B., Zsindely, A., and Berencsi, G. (1967), *Acta Biochim. Biophys. 2 (4)*, 381.
Uziel, M., and Khym, J. X. (1969), *Biochemistry 8*, 3254.
Uziel, M., Koh, C., and Cohn, W. E. (1968,) *Anal. Biochem. 25*, 77.
Uziel, M., Starken, J., Eveleigh, J. W., and Johnson, W. F. (1971), *Clin. Chem. 17*, 740.
Whitfeld, P. R., and Markham, R. (1953), *Nature (London) 171*, 1151.

Immunochemistry of Sperm-Whale Myoglobin. Conformation and Immunochemistry of Derivatives Prepared by Reaction with Diazonium-1*H*-tetrazole. Evaluation of the Specificity of the Reagent†

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ABSTRACT: Sperm-whale myoglobin (Mb) was reacted with diazonium-1*H*-tetrazole at two different pH values. The specificity of each reaction was carefully determined, and the conformational and immunochemical properties of the derivatives were investigated. Studies of the electrophoretically homogeneous derivatives obtained from reaction of Mb with the reagent at pH 6.9 and 8.8 revealed that diazonium-1*H*-tetrazole possessed a marked lack of specificity for histidine or tyrosine even under carefully controlled conditions where, from spectral measurements, one histidine residue was expected to have reacted. Several other amino acids were modified to significant extents as well. Spectral, optical rotatory dispersion, and circular dichroism measurements performed on the derivatives revealed that reaction with the reagent resulted in severe conformational changes relative to Mb. Furthermore, neither derivative exhibited significant antigenic reactivity, relative to the native protein, with antisera directed against MbX. Reaction of human serum albumin with diazonium-1*H*-tetrazole under identical conditions resulted in similar severe modifications of the primary structure and spectral properties of the native protein. The antigenic reactivities of all of these derivatives were further examined employing rabbit antisera prepared against a diazotized derivative of Mb. The results of these investigations revealed that treatment of Mb or human serum albumin with the reagent led to the creation, immunochemically speaking, of new proteins for which the antibody response was directed against new antigenic determinants with the added groups acting as haptens and the nature of the carrier backbones bearing little consequence. Also, diazonium-1*H*-tetrazole is completely nonspecific and totally unsatisfactory as a reagent for selective modification of proteins.

Information concerning the antigenic structure of sperm-whale myoglobin (Mb),[1] reported in several previous communications from this laboratory, represents the most advanced such knowledge for a globular protein (for recent review, see Atassi, 1972). The interaction between *regions* that are close in three-dimensional structure but distant in sequence, to form reactive *sites*[2] has been difficult to investigate. Disruption of such interactions by chemical modification has been useful in yielding some information on this question and most instrumental in the location and delineation of the antigenic reactive region of Mb. This paper reports the detailed immunochemistry as well as the conformations of Mb derivatives prepared by reaction with diazonium-1*H*-tetrazole.

Diazonium-1*H*-tetrazole appeared when introduced (Horinishi *et al.*, 1964) as a very promising reagent that apparently minimized the undesirable nonselective reaction characteristics of common diazonium compounds (Cohen, 1968). This reagent has been applied to differentiate free and bound histidine residues in proteins (Horinishi *et al.*, 1964) and to determine the molar contents of free and iron-linked histidine residues in horse heart and baker's yeast cytochrome *c* (Horinishi *et al.*, 1965). DHT has also been utilized to clarify the role of tyrosine residues in the biological activity of myosin (Shimada, 1970). Several other proteins have been

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* Author to whom correspondence should be addressed. The work was completed during the tenure to M. Z. A. of Established Investigatorship of the American Heart Association.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin: MbX, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964); apoMb, apomyoglobin; DHT, diazonium-1*H*-tetrazole; D-Mb$_7$ and D-Mb$_9$, derivatives prepared by reaction of MbX with DHT at pH 6.9 and 8.8, respectively; HSA, human serum albumin; D-HSA$_7$ and D-HSA$_9$, derivatives prepared by reaction of HSA with DHT at pH 6.9 and 8.8, respectively.

[2] The terms antigenic reactive *regions* and antigenic reactive *sites* are used according to the definitions previously given (Atassi and Saplin, 1968).