Vessey, D. A., and Zakim, D. (1972a), *Biochim. Biophys. Acta 268*, 61.
Vessey, D. A., and Zakim, D. (1972b), *J. Biol. Chem. 247*, 3023.
Wisnes, A. (1972), *Biochim. Biophys. Acta 284*, 392.
Zakim, D., Goldenberg, J., and Vessey, D. A. (1973a), *Biochim. Biophys. Acta 309*, 67.
Zakim, D., Goldenberg, J., and Vessey, D. A. (1973b), *Biochem. Biophys. Res. Commun. 52*, 453.
Zakim, D., and Vessey, D. A. (1972), *Arch. Biochem. Biophys. 148*, 97.
Zakim, D., and Vessey, D. A. (1973), *Methods Biochem. Anal. 21*, (in press).
Zhivkov, V. (1970), *Biochem. J. 120*, 505.

Synthesis and Characterization of Photoaffinity Labels for Adenosine 3′:5′-Cyclic Monophosphate and Adenosine 5′-Monophosphate†

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ABSTRACT: The synthesis and characterization of three ethyl-2-diazomalonyl derivatives of adenosine 3′:5′-cyclic monophosphate ($O^{2'}$-, N^6-, and N^6,$O^{2'}$-di) and of $O^{2'(3')}$-(ethyl-2-diazomalonyl)adenosine 5′-monophosphate are described. These derivatives have potential utility as photoaffinity labels. The N^6 derivatives are subject to pH-dependent Dimroth rearrangements, which imposes some constraints on their utility. From measurements of the rates of rearrangement it is concluded that at room temperature these derivatives can be used at pH 7, while at 5° the useful pH range is extended to pH 8. The rates of rearrangement of ethyl-2-diazomalonyl derivatives of aniline and benzylamine are also measured and are found to be very slow. It is concluded that the Dimroth rearrangement will not pose a general problem for the use of ethyl-2-diazomalonyl derivatives of amines as photoaffinity labels.

Adenosine 3′:5′-cyclic monophosphate (cAMP)[1] appears to play a role in a wide variety of biological processes (Robison *et al.*, 1971) and much current research is devoted to the isolation and characterization of the primary cAMP receptors in cells (Gill and Garren, 1971; Lemaire *et al.*, 1971; Maeno *et al.*, 1971; Walsh *et al.*, 1971; Anderson *et al.*, 1972; Rubin *et al.*, 1972). One approach to this problem is to covalently label the cAMP receptor in the intact cell. The advantages of using photoaffinity reagents for such an approach have been discussed previously (Kiefer *et al.*, 1970; Brunswick and Cooperman, 1971; Knowles, 1972). Two principal points are (1) that the reagents only become reactive on photolysis, so that they may be allowed to become fully equilibrated with the target protein before being activated for covalent bond formation; and (2) that the photolytically generated species are reactive enough to insert into any nearby bond, so that nucleophiles are not required to be present at the site for labeling to be achieved. In this paper we report the synthesis and characterization of three ethyl-2-diazomalonyl derivatives of cAMP of potential use as photoaffinity reagents. Two of these derivatives are substituted on the N^6 position and undergo pH-dependent Dimroth rearrangements. The apparent pK_a and rates of equilibration for this rearrangement have been studied, in order to assess what constraints the rearrangement imposes on the use of the N^6 derivatives as photoaffinity reagents. *N*-(Ethyl-2-diazomalonyl) derivatives of aniline and benzylamine have also been studied to investigate the general importance of the phenomenon for α-diazoamides.

Because of point 2 above, photoaffinity reagents should also be of great utility in characterizing binding sites, such as allosteric sites, even on purified proteins. In this paper we also describe the synthesis and characterization of $O^{2'(3')}$-(ethyl-2-diazomalonyl)adenosine 5′-monophosphate, a potential photoaffinity reagent for adenosine 5′-monophosphate, which is an allosteric effector of a large number of enzymes (Monod *et al.*, 1965).

Studies on the applications of these reagents have been or will be presented elsewhere (Brunswick and Cooperman, 1971; Cooperman and Brunswick, 1973; Guthrow *et al.*, 1973). A preliminary account of part of this work has been published previously (Brunswick and Cooperman, 1971).

Materials and Methods

Adenosine 3′:5′-cyclic monophosphate was obtained from Sigma. [^{3}H]Adenosine 3′:5′-cyclic monophosphate was obtained from New England Nuclear. Beef heart 3′:5′-cyclic nucleotide phosphodiesterase was obtained from Sigma. Ethyl-2-diazomalonyl chloride was prepared as described previously (Brunswick and Cooperman, 1971). *N*-(Ethyl-2-diazomalonyl)aniline was prepared as described by Leffler and Liu (1956). *N*-(Ethyl-2-diazomalonyl)benzylamine was prepared as described by Hoover and Day (1956).

Ascending paper chromatography was performed on either Whatman No. 40 or 3MM (preparative) paper, with an overnight development, using ethanol–0.5 M ammonium acetate (pH 7.0) (5:2, v/v) as solvent. Ascending thin layer chromatography (tlc) was carried out on Macherey-Nagel (Brinckmann)

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[1] Abbreviation used is: cAMP, adenosine 3′:5′-cyclic monophosphate.

PEI-cellulose sheets with 1% LiCl as solvent. Bands were detected by radioactivity and by fluorescence (N^6 derivatives) or by fluorescence quenching. Photolyses were carried out in quartz vessels either in a Rayonet photochemical reactor or with a UVS-11 (Mineralight) lamp. In both cases irradiation was at 253.7 nm. Ultraviolet (uv) absorption spectra were taken with a Cary 15 spectrophotometer, and proton magnetic resonance (pmr) spectra were taken on a Varian HA 100 spectrometer.

All purified compounds showed a single spot on both paper chromatography and tlc.

Preparation of $O^{2'}$-(Ethyl-2-diazomalonyl)adenosine 3′:5′-Cyclic Monophosphate (I). cAMP (0.1 g, 0.3 mmol) and [^{3}H]-cAMP (25 μCi) were dissolved in 3.4 ml of 0.38 M aqueous triethylamine and the solution was evaporated to dryness. The residue was dissolved in dry pyridine and pyridine was removed by evaporation. The dried residue was dissolved with gentle heating and shaking in 2.0 ml of dry pyridine. This solution was brought to 0°, and to it was added, dropwise and with stirring, ethyl-2-diazomalonyl chloride (0.15 ml, 1.14 mmol). Addition took 5 min, after which the reaction mixture was allowed to stand at room temperature for 10 min and then cooled to 0°. Absolute ethanol (2 ml) was added to quench the reaction, and after standing at room temperature for 30 min the solution was evaporated to dryness. The residue was dissolved in two 5-ml portions of absolute ethanol and evaporated to dryness each time. The residue was dissolved in 30 ml of 0.04 M aqueous triethylammonium bicarbonate buffer (pH 7.5), giving a final pH of 5.6, and this solution was chromatographed on a DEAE-cellulose (HCO_3^- form) column (3.4 × 15.0 cm), eluting first with 400 ml of H_2O followed by a linear gradient of triethylammonium bicarbonate at pH 7.2 (0.00–0.07 M, 1.61 total). On this column I is completely resolved from II, which is also formed in the reaction mixture, but is obtained contaminated by small amounts of cAMP and ethyl-2-diazomalonic acid. Both of these impurities are present in part due to partial hydrolysis of I on removal of triethylammonium bicarbonate. Pure samples of I were obtained by preparative paper chromatography. In some cases a second paper chromatography was necessary to remove the last traces of ethyl-2-diazomalonic acid. The final yield of the triethylammonium salt of I was 55 mg (39%). In other preparations the yield varied from 20 to 50%.

Preparation of N^6,$O^{2'}$-Di(ethyl-2-diazomalonyl)adenosine 3′:5′-Cyclic Monophosphate (II). A solution of the dried triethylammonium salt of cAMP (0.5 g, 1.5 mmol; tritiated, 25 μCi) in 26 ml of dry pyridine, prepared as described above, was brought to 0° and to it was added dropwise and with stirring 2.25 ml (17.1 mmol) of ethyl-2-diazomalonyl chloride. Addition took 10 min, after which the reaction mixture was allowed to stand overnight at 4°. To quench the reaction, 11 ml of water was added and the resulting solution was allowed to stand at room temperature for 1 hr before being taken to dryness. The residue was dissolved in two 10-ml portions of absolute ethanol and evaporated to dryness each time. The residue was dissolved in 75 ml of 0.1 M potassium phosphate buffer (pH 7.5) and the solution was brought to pH 5–6 with 1 M NaOH (~10 ml), and extracted with five 50-ml portions of $CHCl_3$. The chloroform extracts were evaporated to dryness, the residue dissolved in 25 ml of 0.04 M triethylammonium bicarbonate (final pH 5.2), and the solution was chromatographed on a DEAE-cellulose (HCO_3^- form) column (4.3 × 23 cm), eluting first with water and then with a linear gradient of triethylammonium bicarbonate (pH 7.2) (0.00–0.15 M, 4 l. total). This column gives the tiethylammonium salt of II in essentially pure form (185 mg, 20%). Small amounts of a non-radioactive impurity were removed by preparative paper chromatography. In two other preparations the yield was 35%.

TABLE I: R_F, λ_{max}, and ϵ_{max} of Derivatives of cAMP and AMP.

Compound	λ_{max}	ϵ_{max}	Photolyzed[a] λ_{max}	Photolyzed[a] ϵ_{max}	R_F[b]	R_F[c]
cAMP	258	14,100			0.35	0.45
N^6-Butyryl-cAMP	272	15,100[d]				
AMP	259	15,400			0.08	0.20
I	254	21,200	258	14,100[e]	0.70	0.45
II	281	32,300[f]			0.70	0.20
	253–256	21,200[f]				
	255	26,000[g]				
	266–269	25,000[g]				
III	281	31,400[f]	273	16,600[f]	0.40	0.15 ± 0.05
	269	24,700[g]				
IV	254	22,000	258	14,800	0.25	0.20

[a] Values for derivatives in which the diazo group was completely photolyzed. For III these values include a small correction for absorption loss due to photolysis of the purine chromophore (see Figure 3). [b] Paper chromatography; solvent system, ethanol–0.5 M ammonium acetate (5:2, v/v). [c] Chromatography on thin-layer PEI; solvent system, 1% LiCl. [d] Falbriard *et al.* (1967). [e] pH 7.0. [f] pH 4.1. [g] pH 9.1.

Preparation of $O^{2'(3')}$-(Ethyl-2-diazomalonyl)adenosine 5′-Monophosphate (IV). The triethylammonium salt of I (17 mg, 0.03 mmol) was dissolved in 1 ml of a solution containing 0.04 M Tris, 0.04 M imidazole, 2 mM $MgSO_4$, and 4 mg (1.2 units) of beef heart cAMP phosphodiesterase (pH 7.4). After 42 hr at room temperature the reaction mixture was subjected to preparative paper chromatography, giving pure IV in high yield (15 mg, 88%).

Preparation of [^{3}H]cAMP Derivatives

$O^{2'}$-[^{3}H](Ethyl-2-diazomalonyl)adenosine 3′:5′-Cyclic Monophosphate (I). [^{3}H]cAMP (0.5 mCi, 0.02 μmol) was treated with ethyl-2-diazomalonyl chloride (15 μl, 0.11 mmol) in 0.2 ml of dry pyridine as described above in the synthesis of I. Tritiated I was purified on a small DEAE-cellulose column and obtained in 30–40% yield.

N^6-[^{3}H](Ethyl-2-diazomalonyl)adenosine 3′:5′-Cyclic Monophosphate (III). [^{3}H]cAMP (0.1 mCi, 0.004 μmol) was treated with ethyl-2-diazomalonyl chloride (15 μl, 0.11 mmol) in 0.2 ml of dry pyridine as described above in the synthesis of II. The reaction was quenched with water and taken to dryness, ethanol was added and evaporated, and the residue was dissolved in 0.4 ml of ice-cold 1 M NaOH. After 4 min, 0.5 ml of 1 M acetic acid was added, and the reaction mixture was paper chromatographed. The radioactive band cochromatographing

TABLE II: Proton Magnetic Resonance Data of Cyclic Nucleotides.[a]

Compound	pD[b]	H-8	H-2	H-1′	H-2′	H-3′	H-4′ + 2H-5′ + OCH_2CH_3
I[c]	6.5	8.32[d] (1.9)	8.32[d] (1.9)	6.44 (0.9)	5.92[e] (1.0)	5.24 (1.3)	4.49[f] (4.8)
II[c]	6.0	8.52 (0.9)	8.69 (1.0)	6.53 and 6.61[g] (1.0)	5.97 (1.0)	5.42 (1.1)	4.50[f] (7.2)
III[c]	4.5	8.42 (1.0)	8.52 (1.1)	6.19 (0.9)	4.80 or 4.84 (1.0 or 1.1)	4.80 or 4.84 (1.0 or 1.1)	4.50[f] (5.1)
cAMP[h]	7.4	8.00 (0.9)	8.06 (1.1)	6.01 (1.0)	4.67[d] (2.3)	4.67[d] (2.3)	4.40[f] (3.0)
$N^6,O^{2'}$-Dibutyryl cAMP[i]		8.39 (1.0)	8.77 (0.9)	6.27 (1.0)	5.81[e] (1.1)	5.29 (1.0)	4.40[f] (2.9)

[a] Chemical shifts are in δ (ppm); numbers in parentheses represent number of protons observed. [b] pH meter reading plus 0.4 (Glasoe and Long, 1960). [c] In D_2O as mixtures of NH_4^+ and Et_3NH^+ salts. [d] Unresolved. [e] Doublets. [f] Apparent center of overlapping multiplets. [g] Two peaks due to presence of both triazole and diazo forms. [h] In D_2O as Na^+ salt. [i] In $CDCl_3$ as Et_3NH^+ salt.

with authentic III was eluted and further purified by tlc, giving tritiated III in 20% yield.

Results and Discussion

Structure Determination. Physical and chemical properties of the adenylate derivatives I–IV are listed in Tables I and II. The structural proof of the cAMP derivatives I–III, starting from cAMP, basically requires knowledge of the number of

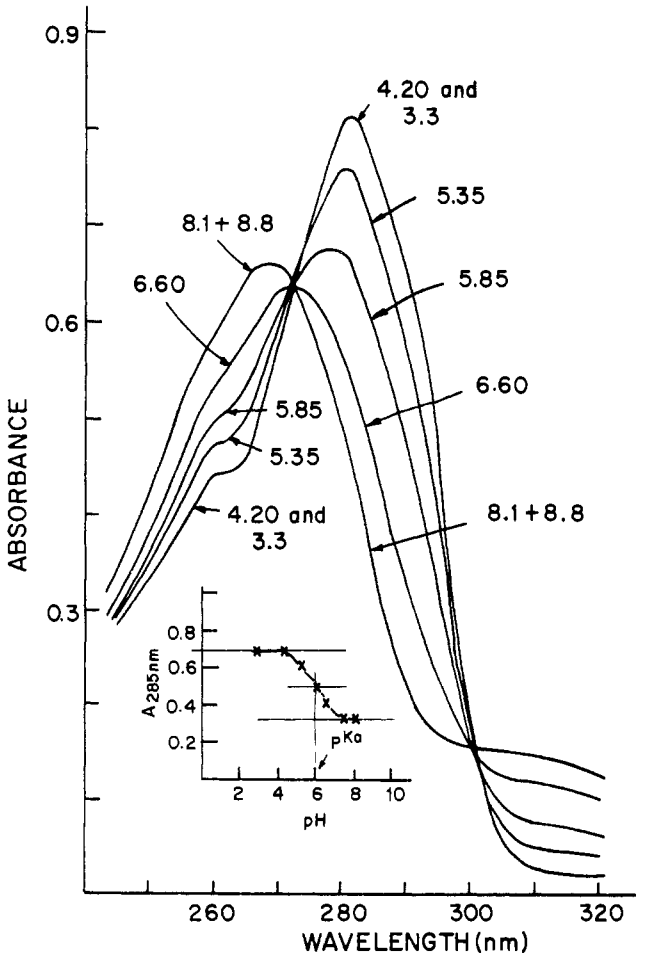
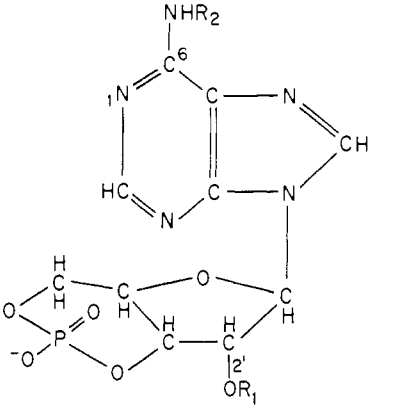
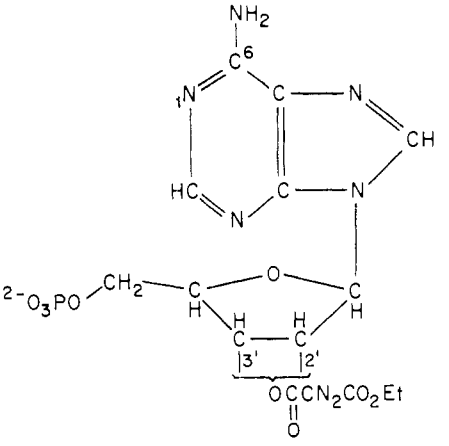


FIGURE 1: Dependence of the uv absorption spectrum of 2.5×10^{-5} M III on pH at 19°. In all cases spectra were taken after full equilibration was achieved; inset, dependence of $A_{285\ nm}$ on pH.



I, $R_1 = -COCN_2CO_2Et$; $R_2 = H$
II, $R_1 = R_2 = COCN_2CO_2Et$
III, $R_1 = H$; $R_2 = -COCN_2CO_2Et$



IV

ethyldiazomalonyl groups attached and their positions of attachment. The number of groups was determined by pmr, by comparison of the integrated intensities of the ethyl and adenosyl peaks, and by uv, by comparison of spectra before and after photolysis. The uv spectrum of I is the sum of two isolated chromophores, the ethyl-2-diazomalonyl group and the parent nucleotide. On photolysis the former absorption is completely lost, and from the change in $A_{255\ nm}$ a stoichiometry of one ethyl-2-diazomalonyl group is indicated. The downfield shift in the position of the 2′-C–H in the pmr spectrum of I as

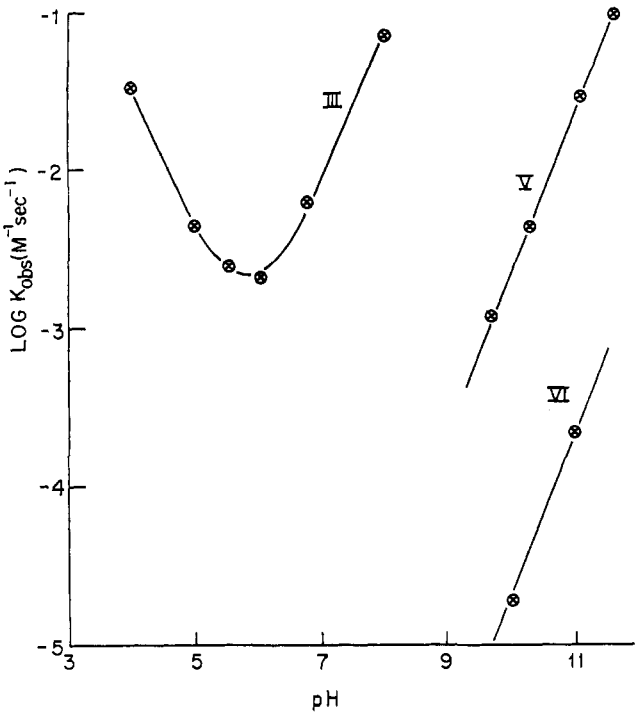


FIGURE 2: Rates of α-diazoamide–triazole equilibration as a function of pH at 19°. For rates measured at pH ≥6, the α-diazoamide forms (III at pH 4.0, V and VI in ethanol) were added to a large excess of buffer and the conversion to triazole was monitored at 280 nm. Rate measurements below pH 6 were obtained similarly, except that III in the triazole form (pH 8) was added to an excess of buffer. Solid lines are calculated using the rate constants given in the text. Points are experimental.

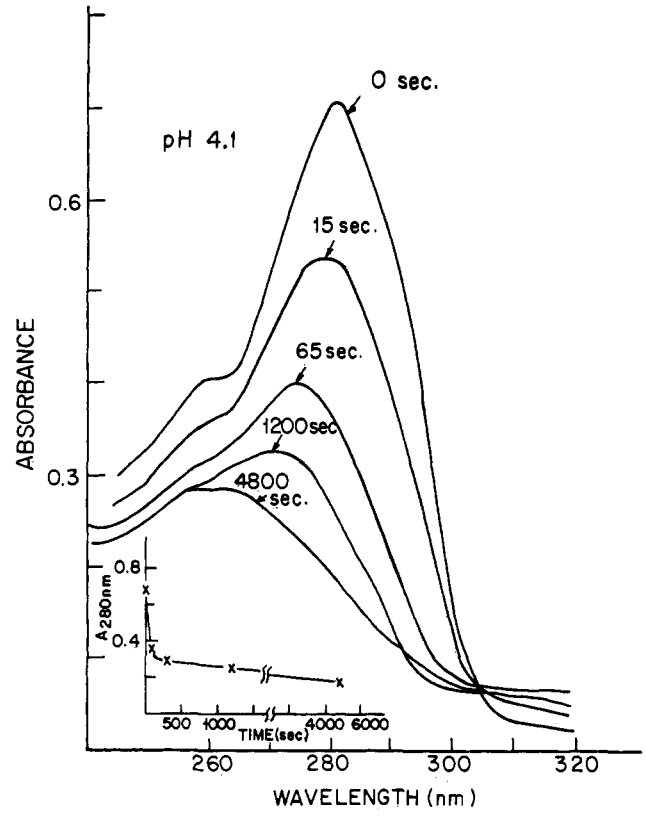


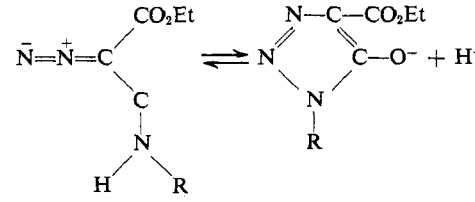
FIGURE 3: Uv absorption spectra of III at pH 4.1 after various times of photolysis; inset, $A_{280\ nm}$ as a function of photolysis time. Photolyses were performed in quartz cuvets taped directly onto an RPR 2537-Å lamp in the reactor with the other lamps removed.

compared with that of cAMP, characteristic of $O^{2'}$-acylated cAMP derivatives (Smith and Jardetzky, 1968), is evidence for linkage of the ethyl-2-diazomalonyl group to the 2′-OH position. Additional evidence is provided by the fact that base treatment of I regenerates cAMP, as shown by chromatographic analysis.

The uv spectrum of III cannot be treated as two separate chromophores so that the pmr results provide the more accurate estimate of ethyl-2-diazomalonyl group stoichiometry. Attachment at N^6 is, however, clearly indicated by the λ_{max} value of the photolyzed compound (273 nm), which is characteristic of N^6-acylated cAMP derivatives (Falbriard *et al.*, 1967). Compound II has the pmr and uv properties to be expected on the basis of the discussions of I and III.

The structure of IV is assigned on the basis of its uv spectrum before and after photolysis, on the fact that base treatment of IV leads to 5′-AMP formation, and on its low R_F on PEI-cellulose tlc plates, characteristic of phosphate monoesters. Griffin *et al.* (1966) have found facile interconversion of $O^{2'}$ and $O^{3'}$ esters of ribonucleosides. For example, for $O^{2'(3')}$-acetyluridine the equilibrium constant for interconversion was 1.7 at pH 7.0, with the $O^{3'}$ isomer favored. We would expect a similar situation to prevail in IV, although we have not as yet determined the equilibrium constant.

Dimroth Rearrangement. The spectrum of III is pH dependent in the neutral pH range, with an apparent spectrophotometric pK_a of 6.0 (Figure 1). This behavior, unusual for an N^6-acylated purine, is due to the formation of a 1,2,3-triazole *via* the Dimroth rearrangement (Dimroth, 1910a,b), which is characteristic of α-diazoamides. The rate at which equilibration is achieved between these two species is strongly dependent on pH. If a solution of III equilibrated at a pH



R = purine 3′:5′-cyclic ribonucleotide

where the diazo (or triazole) form is dominant is rapidly brought to a pH where the triazole (or diazo) form is dominant, the rate of equilibration, measured spectrophometrically, follows pseudo-first-order kinetics. A plot of k_{obsd} as a function of final pH at 19° is given in Figure 2. These data are most simply explained by the reversible reactions 1 and 2, where DH represents the diazo form and T^- is the enolate ion of the 1,2,3-triazole form. k_{obsd} is given by eq 3, where the rate

$$DH + OH^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T^- \quad (1)$$

$$DH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T^- + H^+ \quad (2)$$

$$k_{obsd} = k_1(OH^-) + k_{-1} + k_2 + k_{-2}(H^+) \quad (3)$$

constants have the following approximate values: k_1, 1.21×10^5 M^{-1} sec^{-1}; k_{-2}, 3.3×10^2 M^{-1} sec^{-1}; $k_{-1} + k_2$, 1.0×10^{-3} sec^{-1}. Since k_2/k_{-2} is equal to $K_w(k_1/k_{-1})$, we can further obtain approximate values for k_{-1}, 0.5×10^{-3} sec^{-1}, and k_2, 0.5×10^{-3} sec^{-1}. Over the whole pH range in which rates were measured (4–9), there was no evidence in the uv spectrum

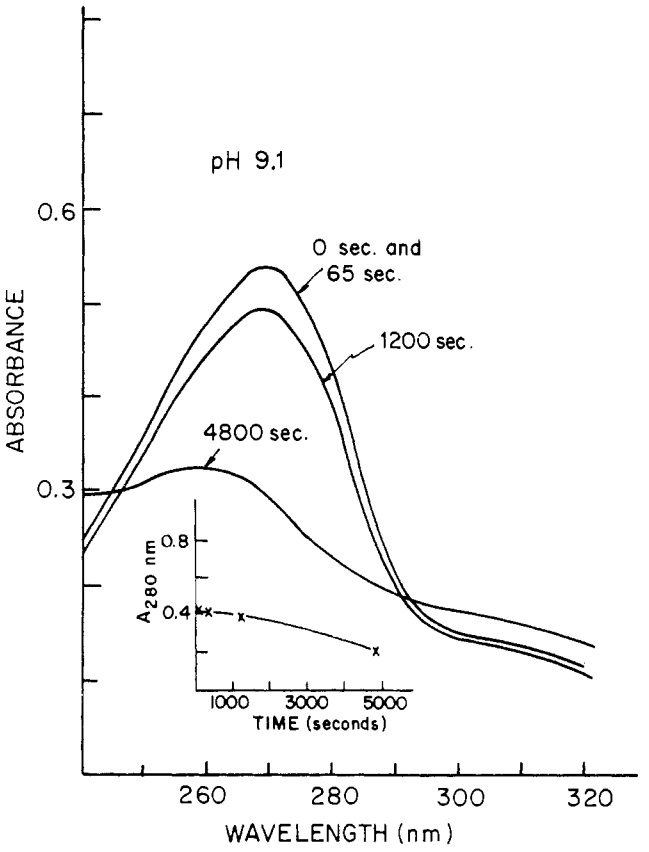


FIGURE 4: Uv absorption spectra of III at pH 9.1 after various times of photolysis; inset, $A_{280\ nm}$ as a function of photolysis time. Photolyses were performed in quartz cuvets taped directly onto an RPR 2537-Å lamp in the reactor with the other lamps removed.

for the formation of a third species, either TH or D^-, so that the true pK_a of TH must be fairly acidic (<4) while that of DH must be quite basic (>9).

Photolysis of III at pH 4.1 (diazo form) results in rapid loss of diazo group absorption, followed by a slow change in the purine spectrum (Figure 3). This slower process appears to be characteristic of N^6-acylated purines, since N^6-butyryl-cAMP behaved similarly while cAMP and I showed no such change. Photolysis of III at pH 9.1 (triazole form) results in a slow loss of triazole and purine absorption (Figure 4).

Failure of the triazole form of III to efficiently photolyze to yield a carbene poses a problem for practical use of III as a cAMP photoaffinity label, at least at pH values >6.5–7.0, where the triazole form predominates. In some cases this problem could be overcome by adding III in the diazo form to a solution of pH >7 and photolyzing before isomerization to the triazole becomes important. At 19°, $t_{1/2}$ for equilibration is 90 sec at pH 7 and 10 sec at pH 8. However, at 5°, $t_{1/2}$ is 140 sec at pH 8, thus increasing the useful pH range. In our apparatus $t_{1/2}$ for photolysis of 0.02 mM III (diazo form) is about 20 sec.

In order to assess whether the Dimroth rearrangement would be a general problem for the use of α-diazoamides as photoaffinity reagents, the rates of isomerizations of *N*-(ethyl-2-diazomalonyl)aniline (V) and *N*-(ethyl-2-diazomalonyl)-benzylamine (VI) to the corresponding 1,2,3-triazoles were measured (Figure 2). The rate constants for the hydroxide ion dependent (corresponding to k_1 above) isomerizations are 37 M^{-1} sec^{-1} for V (19°) and 0.21 M^{-1} sec^{-1} for VI (25°), so that in the neutral pH range the Dimroth rearrangements should not be a problem for ethyl-2-diazomalonyl derivatives of amines. III is an ethyl-2-diazomalonyl derivative of an amidine nitrogen, and this may account for its differences from V and VI.

Although we have discussed the negative aspects of the Dimroth rearrangement, it is well to point out one potential positive aspect as well. As would be anticipated, the behavior of II is similar to that of III, with a pK_a for the Dimroth rearrangement of 6.4. Thus, it might be possible to use II as a cross-linking reagent, photolyzing the O^2-(ethyl-2-diazomalonyl) group selectivity at high pH to give incorporation into one cAMP-binding macromolecule, then lowering the pH and photolyzing the N^6-(ethyl-2-diazomalonyl) group in the presence of a second cAMP-binding macromolecule.

Acknowledgment

We would like to thank Mr. Milan Smolko and Mr. Banchong Tangsutichai, who prepared the ethyl-2-diazomalonyl derivatives of aniline and benzylamine, and who did preliminary kinetic studies.

References

Anderson, W. B., Perlman, R. L., and Pastan, I. (1972), *J. Biol. Chem. 247*, 2717.

Brunswick, D. J., and Cooperman, B. S. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1801.

Cooperman, B. S., and Brunswick, D. J. (1973), *Biochemistry 12*, 4079.

Dimroth, O. (1910a), *Justus Liebigs Ann. Chem. 373*, 131.

Dimroth, O. (1910b). *Justus Liebigs Ann. Chem. 377*, 349.

Falbriard, J. G., Posternak, T., and Sutherland, E. W. (1967), *Biochim. Biophys. Acta 148*, 99.

Gill, G. N., and Garren, L. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 786.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Griffin, B. E., Jarman, M., Reese, C. B., Sulston, J. E., and Trentham, D. R. (1966), *Biochemistry 5*, 3638.

Guthrow, C. E., Rasmussen, H., Brunswick, D. J., and Cooperman, B. S. (1973), *Proc. Nat. Acad. Sci. U. S.* (in press).

Hoover, J. R. E., and Day, A. R. (1956), *J. Amer. Chem. Soc. 78*, 5832.

Kiefer, H., Lindstrom, J., Lennox, E. S., and Singer, S. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1688.

Knowles, J. R. (1972), *Accounts Chem. Res. 5*, 155.

Leffler, J. E., and Liu, S.-K. (1956), *J. Amer. Chem. Soc. 78*, 1949.

Lemaire, S., Pelletier, G., and Labrie, F. (1971), *J. Biol. Chem. 246*, 7303.

Maeno, H., Johnson, E. M., and Greengard, P. (1971), *J. Biol. Chem. 246*, 134.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971), Cyclic AMP, New York N. Y., Academic Press.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972), *J. Biol. Chem. 247*, 6135.

Smith, M., and Jardetzky, C. D. (1968), *J. Mol. Spectrosc. 28*, 70.

Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., and Krebs, E. G. (1971), *J. Biol. Chem. 246*, 1968.