on the protein carrier. The positive serum titer was approximately 1 x 10^{-7} with a 50% positive response at approximately 5 x 10^{-6}, which was the antiserum dilution selected for subsequent inhibition studies. Similar results were observed for antiserum collected from rabbit A1. No significant change in the titer of the antiserum was observed in bleeds "Ab" or "Ac" for either rabbit.

B. Specificity of the Antiserum

Attempts to competitively inhibit anti-BSA-(S) 8,5'-cycloAdo antibodies from BSA-(S) 8,5'-cycloAdo adsorbed on to the microtiter wells were unsuccessful with either the hapten [(S) 8,5'-cycloAdo] or 5'-AMP. However, a serial dilution of the BSA-(S) 8,5'-cycloAdo conjugate in free solution effectively competed for antibody binding sites as illustrated in Figure 20. The inability of this antiserum to

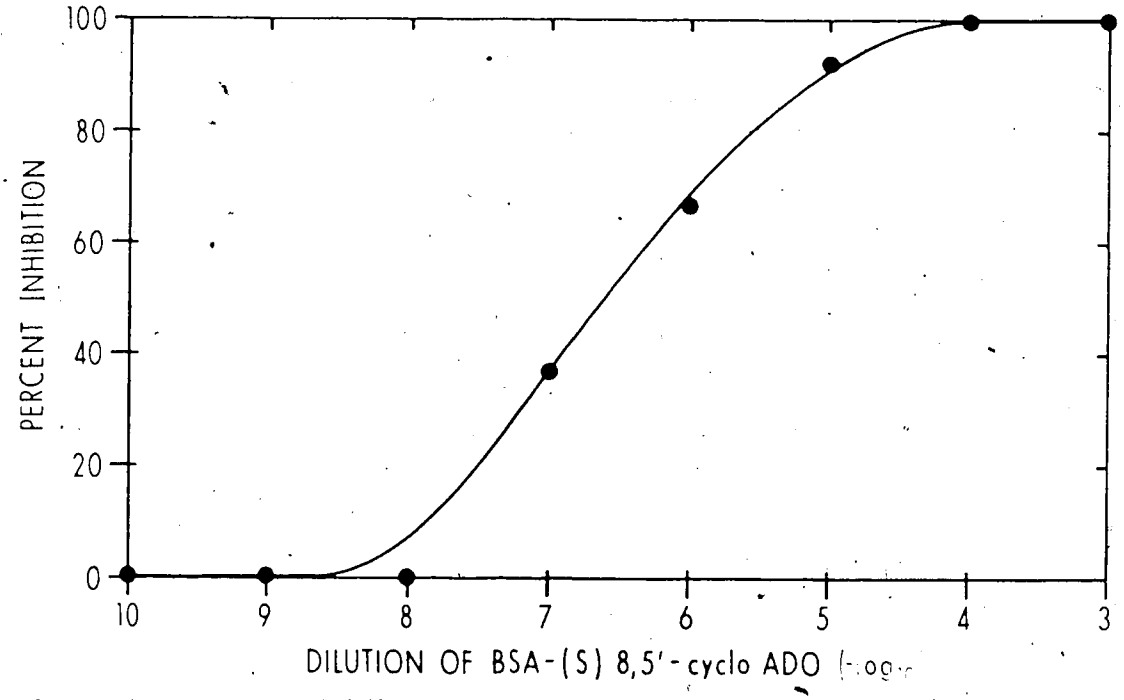

FIGURE 20: ELISA inhibition assay for anti-BSA-(S) 8,5'-cycloAdo antiserum. (rabbit A2, "Aa" bleed.) BSA-(S) 8,5'-cycloAdo was used to coat the microtiter plate wells and as a competitive inhibitor for the assay. Anti-BSA antibodies were adsorbed by the addition of 1% BSA. The antiserum was diluted to 5 x 10^{-6}.

recognize (S) 8,5'-cycloAdo in free solution prevented subsequent use of these antibodies as reagents for ELISA. The response of both immunized rabbits was similar.

II. ANTIBODIES RAISED TO HEMOCYANIN-Im(R) 8,5'-cycloAMP

A. Reagent Dilution Assay

Antiserum from rabbit R001 ("re" bleed) was screened using the BSA-Im (S) 8,5'-cycloAMP conjugate to coat the wells of microtiter plates (Figure 21). The use of a BSA conjugate as opposed to a hemocyanin conjugate, was necessitated due to precipitation of hemo-

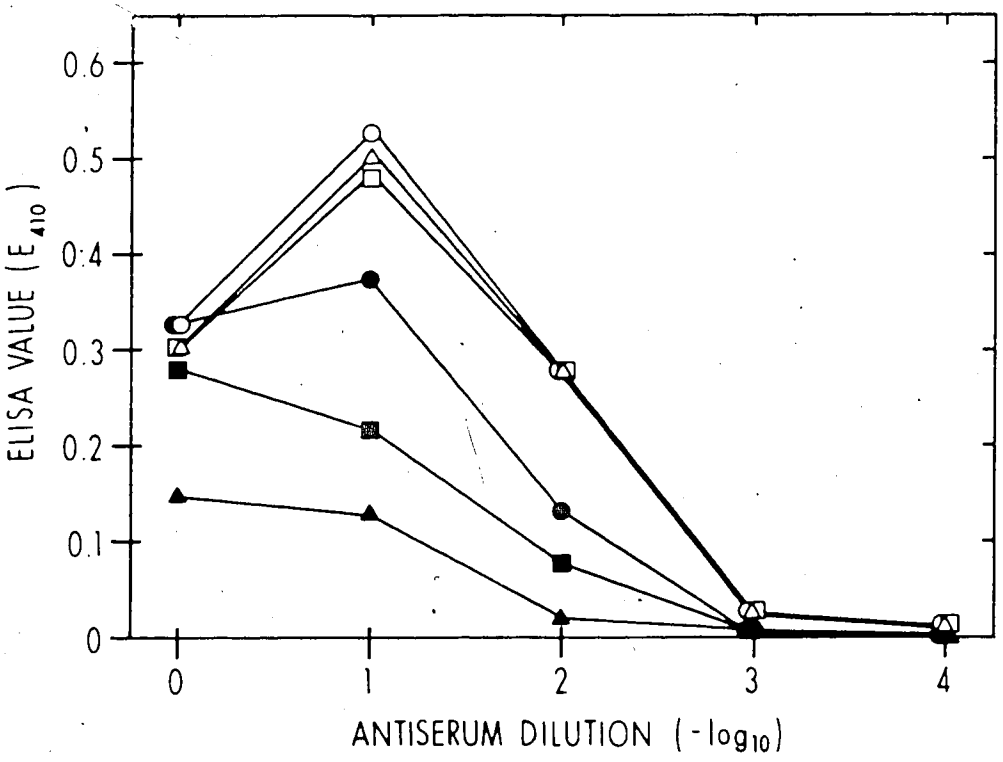

FIGURE 21: Reagent dilution assay for anti-hemocyanin-Im(R) 8,5'-cycloAMP antiserum.[a] (Rabbit R001, "re" bleed.) Antiserum containing anti-hemocyanin-Im(R) 8,5'-cycloAMP antibodies was screened with microtiter plates coated with BSA-Im(S) 8,5'-cycloAMP in an ELISA. (Dilution of conjugate in coating buffer: △ 10^{-1}, □ 10^{-2}, ○ 10^{-3}, ● 10^{-4}, ■ 10^{-5} and ▲ 10^{-6}.)

cyanin - anti-hemocyanin antibodies when hemocyanin - Im (R) 8,5'-cycloAMP was used to coat the wells. (The (S) epimer was used due to limited quantities of the (R) epimer). The optimal dilution of the BSA-Im (S) 8,5'-cycloAMP conjugate for coating the polyvinyl chloride plates was 1×10^{-3}, corresponding to a conjugate concentration of 10 µg/mL. Plates coated with 500 µg/mL BSA showed no significant reactivity with the antiserum. The titer of the antiserum was 1×10^{-3}. The dilution at which a 50% positive response occurred was at 1×10^{-2}, the dilution at which subsequent inhibition studies were performed. No significant change in the titer of the antiserum was observed after one further immunization and one bleed one month later. The second rabbit (R002) died, possibly due to complications arising from cardiac puncture, though the animal did not appear traumatized initially following the operation.

B. Specificity of the Antiserum

The concentration of various nucleosides, nucleotides and bases required to inhibit the binding of the antiserum to immobilized BSA-Im (S) 8,5'-cycloAMP by 50% was determined (Figure 22). Inhibition of the anti-hemocyanin - Im (R) 8,5'-cycloAMP was most effective with the immunizing hapten-(R) 8,5'-cycloAMP. Table 7 compares the specificity of the antiserum by listing the IC_{50} (the concentration (µM) required for 50% inhibition of the antiserum) as calculated from Figures 22, and the index of dissimilarity (ID) (calculated by dividing the IC_{50} of the sample by the IC_{50} for the immunizing hapten) (Prager and Wilson, 1971). The concentration required for 50% inhibition (IC_{50}) for the (R) epimer was 28 nM and 250 nM for the (S) epimer. The IC_{50} for 5'-AMP and 5'-dAMP

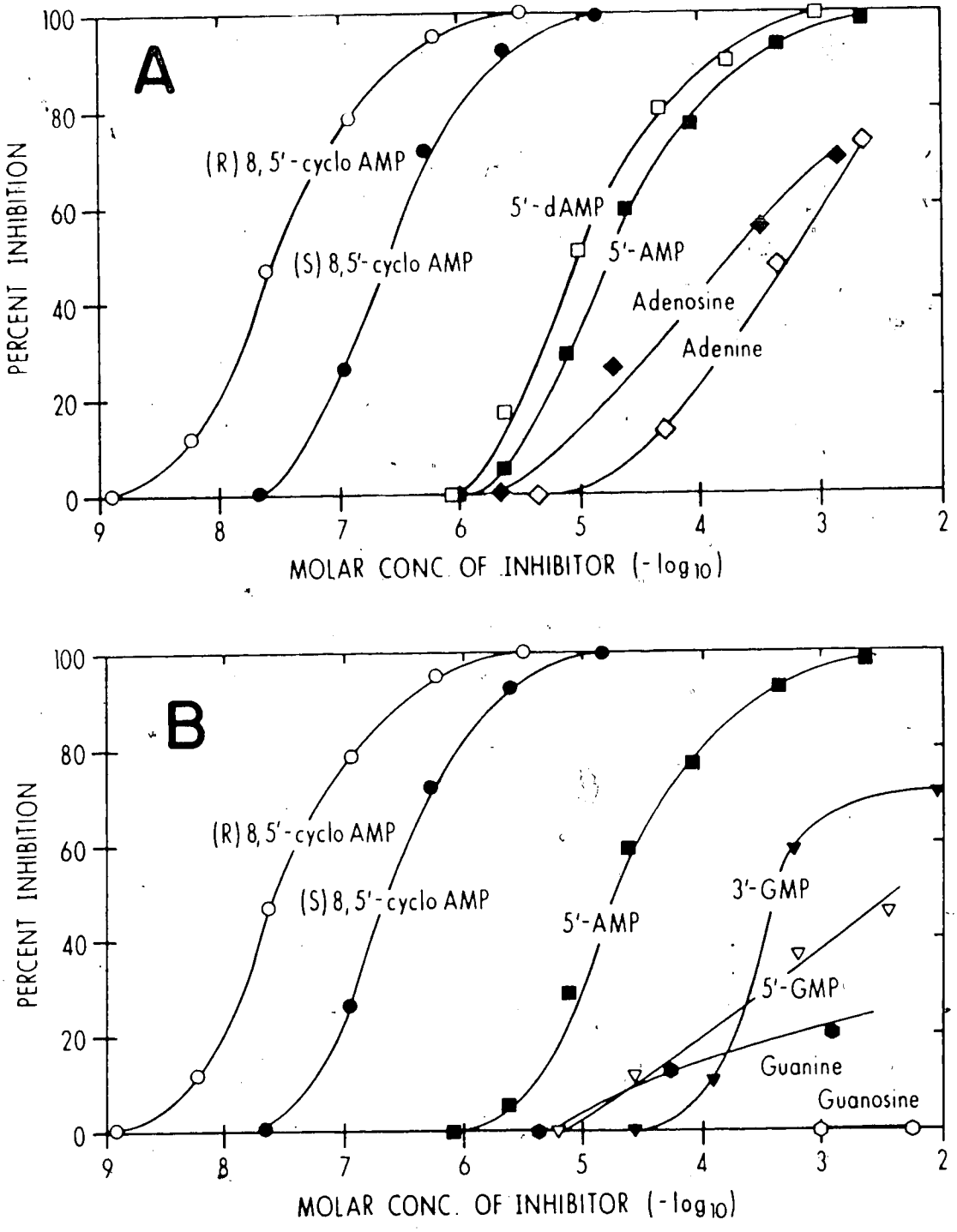

FIGURE 22 (A AND B): Competitive inhibition curves for anti-hemocyanin-Im(R) 8,5'-cycloAMP antiserum. (rabbit R001, "re" bleed). Dilutions of various compounds were preincubated with antiserum at a final dilution of 1×10^{-2}, then added to microtiter wells precoated with 10 µg/ml of BSA-Im(S) 8,5'-cycloAMP for competitive ELISA. Immunization was performed with a hemocyanin-Im(R) 8,5'-cycloAMP conjugate.

TABLE 7: REACTIVITY OF ANTI-HEMOCYANIN-Im(R) 8,5'-cycloAMP ANTISERUM

HAPTEN: (R) 8,5'-cycloAMP

RABBIT: R001. "re" bleed

	IC_{50} [a]	ID [b]
(R) 8,5'-cycloAMP	0.028	1.00
(S) 8,5'-cycloAMP	0.250	8.93
5'-AMP	19	670
5'-dAMP	10	357
3'-AMP	190	6786
Ado	190	6786
2'-dAdo	3500	125,000
Ade	550	19,643
5'-GMP	3500	125,000
3'-GMP	420	15,000
Guo	7000	250,000
Gua	7000	250,000

[a] IC_{50} is the concentration (μM) required for 50% inhibition of the antiserum.

[b] ID is the index of dissimilarity that is calculated by dividing the IC_{50} of the sample by the IC_{50} for the immunizing hapten (R) 8,5'-cycloAMP (Prager and Wilson, 1971).

was 10 μM and 10 μM respectively. The contribution of the 2'-OH to the specificity of the antiserum is therefore minimal. The position of the phosphate group is significant as demonstrated by the observation that the IC_{50} for 3'-AMP is 100 times greater than that for 5'-AMP and the IC_{50} of adenosine, which is also 100 times greater than that of 5'-AMP. Loss of both the 2'-OH group and the phosphate group, as illustrated with 2'-Ado, results in a significant increase in the IC_{50} to 3500 μM. Adenine exhibits an IC_{50} of 550 μM which is unexpectedly low considering the specificity of the antiserum to 2'-dAdo. Cross-reactivity of the antiserum with 5'-GMP, guanosine and guanine, is insignificant. The IC_{50} for 3'-GMP is 420 μM, which is unexpectedly low for a compound containing guanine as the base and no explanation for this data is offered.

C. Effect of Increasing Concentrations of 5'-AMP on the Specificity of the Antiserum

The measurement of 8,5'-cycloAMP in the presence of a relatively large concentration of 5'-AMP is one possible scenario for detection of the hapten in irradiated nucleic acids. This necessitated an investigation into the effects of increasing concentrations of 5'-AMP in the presence of 8,5'-cycloAMP. Antiserum from "rc" bleed was used as a reagent to assay 8,5'-cycloAMP. Figure 22 indicates that the present ELISA methodology is sufficiently sensitive to detect nanomolar quantities of the (R) epimer without significant cross-reactivity in the presence of micromolar concentrations of 5'-AMP. To confirm these preliminary data, a competitive inhibition study was conducted to detect the (R) and (S) epimer in the presence of 10.0, 2.3 and 0.60 μM 5'-AMP. Figure 23 illustrates that 10.0 μM 5'-AMP is sufficient to

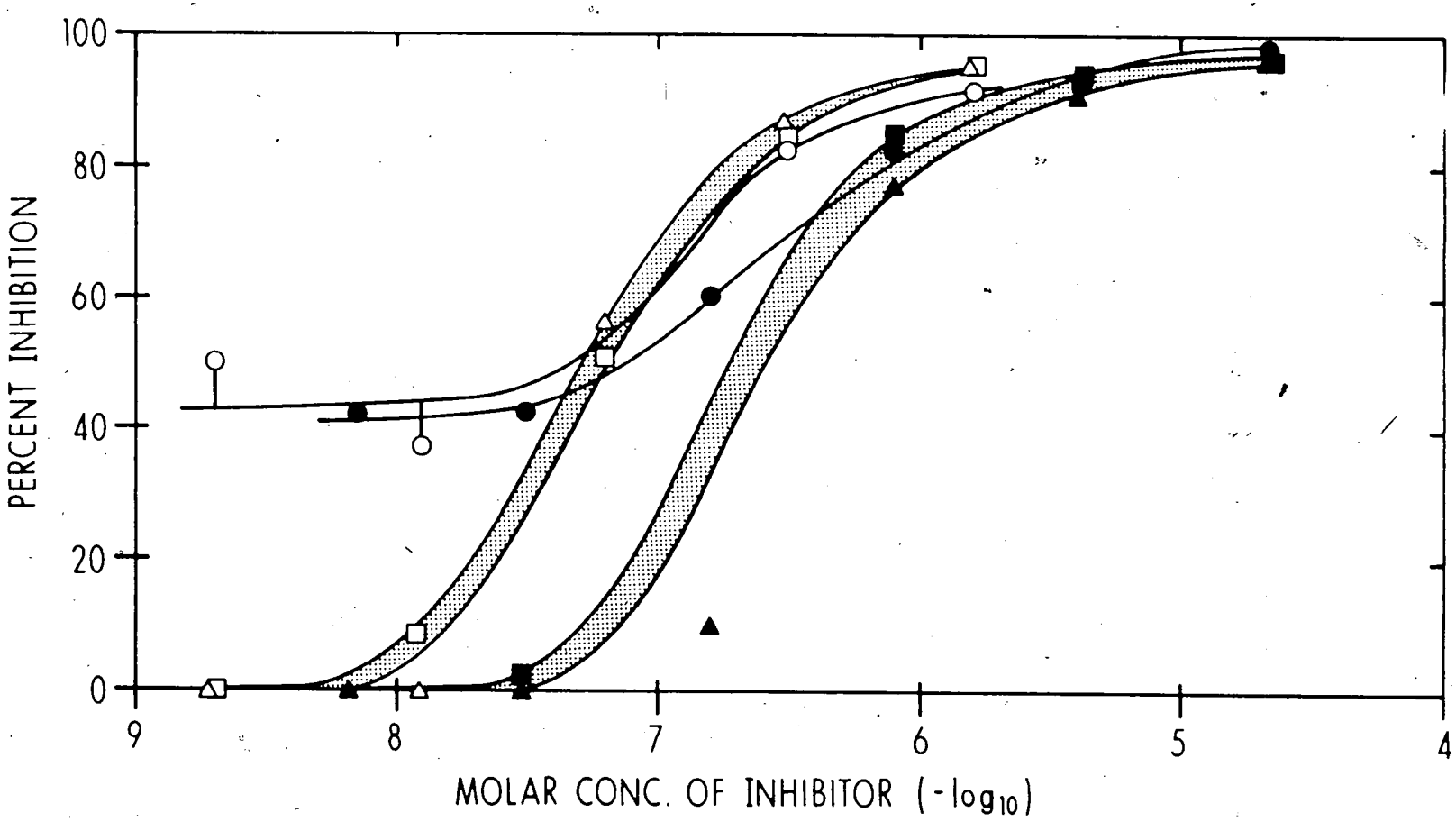

FIGURE 23: Effect of increasing concentrations of 5'-AMP on the specificity of the antiserum (rabbit R001, "rc" bleed). Competitive inhibition curves for the (R) epimer (open symbols) and the (S) epimer (closed symbols) of 8,5'-cycloAMP in the presence of 0.69 (△ , ▲), 2.3 (□ , ■) or 16.0 (○ , ●) μM 5'-AMP.

alter the inhibition curves for the (R) and (S) epimer and that this concentration of 5'-AMP can efficiently compete for 40% of the antibody binding sites. On the other hand, 2.27 or 0.60 μM concentrations of 5'-AMP do not significantly alter the shapes of the inhibition curves. The IC_{50} for the (R) and (S) epimers is not significantly altered in the presence of 2.27 or 0.60 μM 5'-AMP from those reported in the absence of 5'-AMP [(R) epimer IC_{50} 0.055 μM; (S) epimer IC_{50} 0.220 μM].

D. Dose-Yield Response in the Formation of 8,5'-cycloAMP from Irradiated Solutions of 5'-AMP

The molar concentration of (R) 8,5'-cycloAMP and 5'-AMP is plotted as a function of the ELISA value (E_{410}) in Figure 24. Regression analysis of the data for the (R) epimer yields the equation: $y = 0.19x + 0.02$ with a correlation coefficient of 0.0014, and for 5'-AMP: $y = 0.22x + 0.58$ with a correlation coefficient of 0.0048. There is minimal cross-reactivity with the antiserum if the concentration of 5'-AMP is less than approximately 1.0 μM. Using the calibration curve for the (R) epimer, the concentration of 8,5'-cycloAMP from the irradiated samples may be calculated from the ELISA reading. The lower limit of detectability of 8,5'-cycloAMP is 2.0 nM.

The formation of 8,5'-cycloAMP from irradiated solutions of 5-AMP was assayed with this ELISA. The concentration of 8,5'-cycloAMP is plotted as a function of the dose of radiation for nitrous oxide saturated solutions of 5'-AMP irradiated at pH 8.0 (Figure 25). The formation of 8,5'-cycloAMP may be described by the linear relationship: $y = 0.21x - 7.02$ with a correlation coefficient of 0.0720. Formation of 8,5'-cycloAMP may be detected following a total dose of 330 Gy.

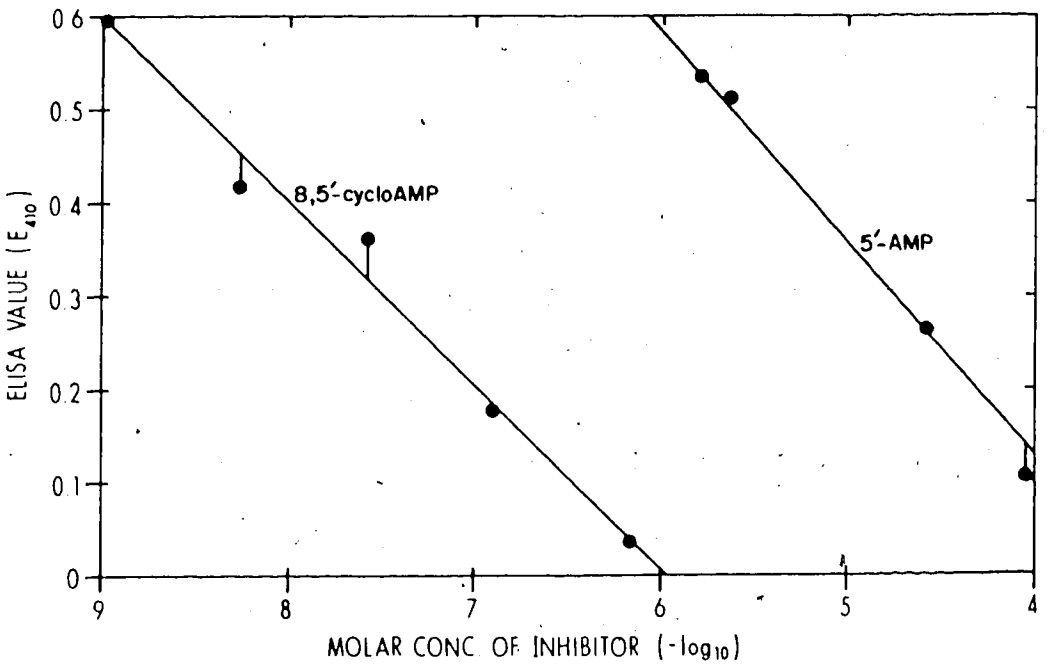

FIGURE 24: Calibration curve for 8,5'-cycloAMP (Antiserum: rabbit R001, "re" bleed.

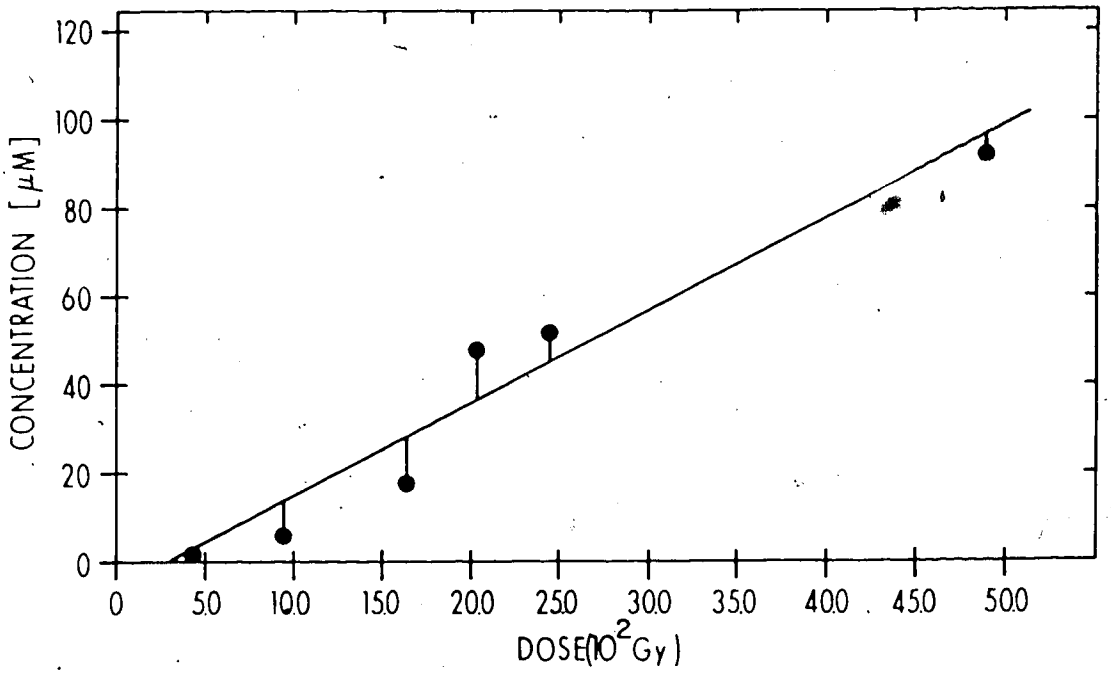

FIGURE 25: Formation of 8,5'-cycloAMP in irradiated solutions of 5'-AMP. Millimolar solutions of 5'-AMP in deionized H_2O were adjusted to pH 8.9 with dilute sodium hydroxide, saturated with nitrous oxide and irradiated at 79.8 Gy/min to total doses between 0 and 5000 Gy. Following irradiation, samples were diluted 1000-fold and assayed by ELISA techniques. Formation of 8,5'-cycloAMP was detected at 330 Gy with this ELISA.

III. ANTIBODIES RAISED TO BSA-Im (S) 8,5'-cycloAMP

A. Reagent Dilution Assay

Antiserum from rabbit S003 ("sb" bleed) was screened by coating the microtiter plates with BSA-Im (S) 8,5'-cycloAMP. Antibodies which recognize determinants on the carrier protein were adsorbed with 1% BSA in all ELISAs. This adsorption of anti-BSA antibodies was sufficient to prevent significant binding of the antiserum to wells coated with 500 µg ml BSA in the presence or absence of competitors. The optimal dilution of BSA-Im (S) 8,5'-cycloAMP for coating polyvinyl chloride plates was 1×10^{-3} corresponding to a conjugate concentration of 10 µg ml (Figure 26). The titer of the antiserum was approximately 1×10^{-6}. The dilution at which a 50% positive response occurred was 5.0×10^{-4} which was the antiserum dilution used for the inhibition assay. The second rabbit (S004) did not respond with specific antiserum and an inhibition assay was not performed.

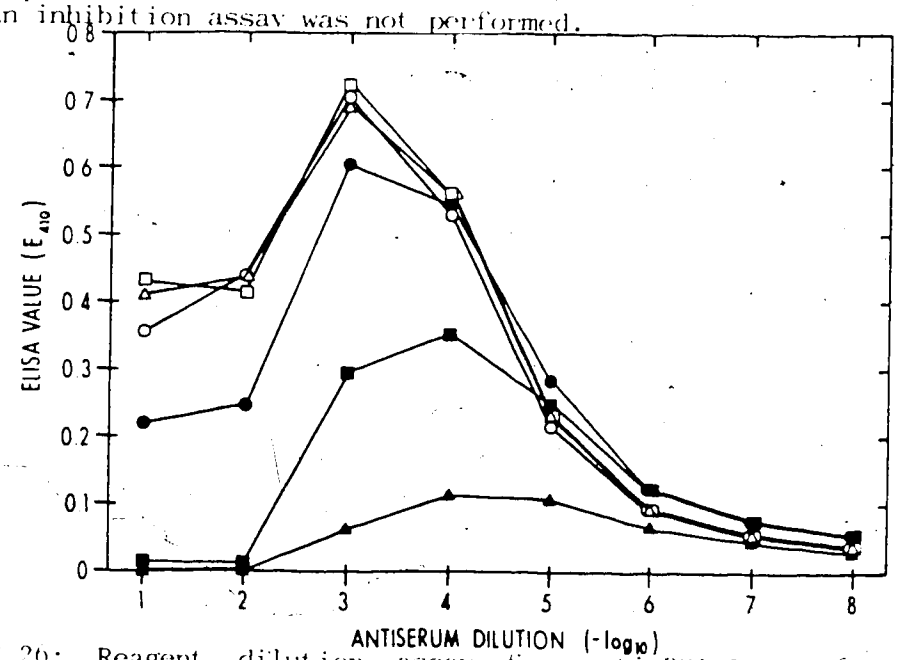

FIGURE 26: Reagent dilution assay for anti-BSA-Im(S) 8,5'-cycloAMP antiserum. (rabbit S003, "sb" bleed.) Antiserum containing anti-BSA-(S) 8,5'-cycloAMP antibodies was screened with microtiter plates coated with BSA-Im(S) 8,5'-cycloAMP in an ELISA. (Dilution of conjugate in coating buffer; △ 10^{-1}, □ 10^{-2}, ○ 10^{-3}, ● 10^{-4}, ■ 10^{-5}, and ▲ 10^{-6}.)

B. Specificity of the Antiserum

The concentration of various nucleotides, nucleosides and bases required to inhibit the binding of antisera to immobilized BSA-Im (S) 8,5'-cycloAMP by 50% was determined from Figure 27 and is listed in Table 8. Inhibition of the anti-BSA-Im (S) 8,5'-cycloAMP antibodies was only slightly more effective with the immunizing hapten - (S) 8,5'-cycloAMP (IC_{50} = 400 nM) than with the (R) epimer (IC_{50} = 750 nM). The plateauing of these and subsequent inhibition curves reflects the antibody heterogeneity of this antiserum.

The specificity of S003 antiserum was substantially different than that of R001 antiserum. One clear difference between the antisera is the similar IC_{50}'s of the (S) and (R) epimers of 8,5'-cycloAMP with S003 antiserum. Secondly, the 2'-OH group does not appear to be a major determinant recognized by antibodies present in R001 serum. However, with S003 antiserum, 5'-AMP has a lower IC_{50} than that for 5'-dAMP, suggesting that the 2'-OH group is partially recognized as an antigenic determinant. Loss of the phosphate determinant as exemplified by adenosine (IC_{50} greater than 10,000) and the base adenine (IC_{50} greater than 10,000) are also significant deviations from the basic structure of the hapten that is recognized by the antiserum. Guanine, guanosine 5'-GMP and 3'-GMP show no significant cross-reactivity with the S003 antiserum.

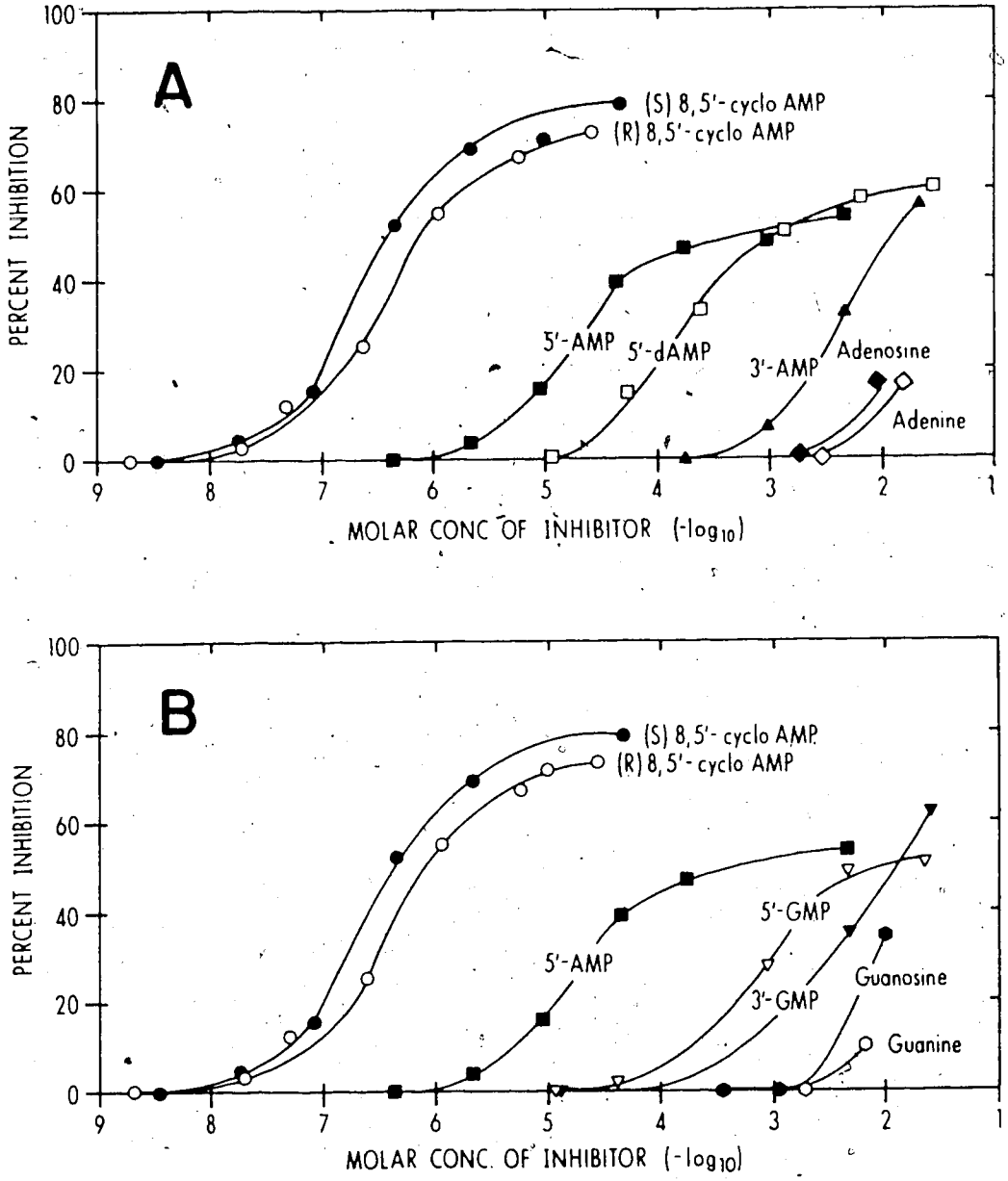

FIGURE 27 (A AND B): Competitive inhibition curves for anti-BSA-Im(S) 8,5'-cycloAMP antiserum. (rabbit S003, "sb" bleed). Dilutions of various compounds were preincubated with antiserum at a final dilution of 5×10^{-4}, then added to microtiter wells precoated with 10 µg/mL of BSA-Im(S) 8,5'-cycloAMP for competitive ELISA. Immunization was performed with a BSA-Im(S) 8,5'-cycloAMP conjugate. Antibodies to BSA were adsorbed with 1% BSA added to the primary antiserum solution.

TABLE 8: REACTIVITY OF ANTI-BSA-Im(S) 8,5'-cycloAMP ANTISERUM

HAPTEN: (S) 8,5'-cycloAMP

RABBIT: S003, "sb" bleed

	IC_{50}[a]	ID[a]
(S) 8,5'-cycloAMP	0.40	1.0
(R) 8,5'-cycloAMP	0.75	1.88
5'-AMP	40	100
5'-dAMP	400	1000
3'-AMP	4000	10,000
Ado	10,000	25,000
Ade	10,000	25,000
5'-GMP	1000	2500
3'-GMP	4000	10,000
Guo	8000	20,000
Gua	6000	15,000

[a] IC_{50} and ID are defined in footnotes a and b of Table 7.

DISCUSSION

The present work describes the development of an ELISA assay with specificity to the radiation-induced intramolecular cyclization of adenosine-5'-monophosphate, which leads to the formation of both diastereoisomers of 8,5'-cycloadenosine-5'-monophosphate. Immunochemistry offers inherent advantages for the detection of radiation-induced chemical modifications. This thesis demonstrates the high specificity inherent in immune reactions which enable antibodies to act as probes for radiation-specific chemical modifications and the high sensitivity of detection for these chemical modifications.

The development of this ELISA assay was complicated by the inability of the immunizing hapten-(S) 8,5'-cycloadenosine to competitively inhibit binding of the antibodies raised against determinants on the BSA-(S) 8,5'-cycloadenosine conjugate. This observation may reflect the possibility that the antibody binding sites with the greatest avidity may recognize determinants contributed by both the hapten and protein, or that the primary determinant recognized by the antibodies does not include the 8,5'-cycloAdo moiety. Further, periodate oxidation of (S) 8,5'-cycloAdo results in cleavage of the C(2') to C(3') bond which may lead to a relaxation of the heterocyclic ring defined by C(1') - O - C(4') - C(5') - C(8) - N(9). The resultant antibodies raised to the periodate-oxidized analogue may possess a substantially different binding site due to conformational changes induced in the (S) 8,5'cycloAdo moiety. This latter hypothesis may be resolved by synthesizing the morpholine derivative of (S) 8,5'-cycloAdo by the method of Khym (1963) and utilizing this species as an inhibitor in a competitive ELISA.

Conjugation of the (R) 8,5'-cycloAMP moiety to hemocyanin through the phosphate group by the imidazolide method (Johnston et al., 1983) provided a useful immunogen to elicit polyclonal antibodies with specificity to the 8,5'-cycloAMP determinant. Although the specificity and sensitivity of the ensuing ELISA assay was satisfactory, the antibody concentration was unexpectedly low. Spectrophotometric measurements of the hemocyanin-Im(R) 8,5'-cycloAMP conjugate were not attempted due to the inability to dissolve this conjugate in an appropriate solvent. The low concentration of specific antibodies to the hapten as evidenced by the requirement of a high concentration of antiserum for the assay, may therefore be a result of a low molar substitution ratio. Hemocyanin was selected as the protein carrier because of suggestions that it could lead to an increased immunogenicity as compared to BSA (Kruger and Gershon, 1972; Rainen and Stollar, 1978). The low concentration of specific antibodies may also be attributed to a poor response of the individual rabbits (see Chapter 2: INTRODUCTION). That 8,5'-cycloAMP can elicit a significant immune response is evidenced by the high titer of specific antibodies produced upon immunization with an immunogen containing approximately 2 molecules of (S) 8,5'-cycloAMP per molecule of BSA.

As probes for the detection of chemical modifications, immunoassays provide exceptional specificity. The ELISA assay developed herein with the hemocyanin - Im (R) 8,5'-cycloAMP conjugate for which the titer of the antiserum was measured at a dilution of 1×10^{-3}, demonstrates that the (R) diastereoisomer of 8,5'-cycloAMP (ID = 1.0) may be resolved from the (S) diastereoisomer (ID = 9.0), the parent

compound 5'-AMP (ID 0800), 5'-dAMP (ID 3000) and the nucleoside, adenosine (ID 08000). In a subsequent study, antiserum was raised utilizing a BSA - Im (S) cycloAMP conjugate. There was a significant increase in the titer of the antiserum to 1×10^{-6}, but this was not paralleled by a significant change in the specificity of the antiserum produced. (S) 8,5'-CycloAMP (ID 1.0) was not well resolved from (R) 8,5'-cycloAMP (ID 1.88), but was sufficiently different from 5'-AMP (ID greater than 100) or 5'-dAMP (ID greater than 1000). The specificity of the antiserum did not significantly increase following subsequent immunizations and bleedings (Fuciarelli and Raleigh, unpublished observations) and, apparently, did not increase upon a 1000 fold increase in the antibody titer. However, the selection of different carrier proteins and epimers certainly complicates evaluation of the relationship between titer and specificity.

The sensitivity of the ELISA assay may be compared with methods previously used to detect 8,5'-cycloAMP in irradiated solutions of 5'-AMP. High pressure liquid chromatography has a reproducible lower limit of detectability for this species of approximately 5 μM (Fuciarelli and Raleigh, unpublished observations). The threshold of detectability of the related 8,5'-cycloAdo moiety in a phage neutralization assay was estimated at 0.5 μM (Lewis and Ward, 1978). In comparison, the limit of detectability of (R) 8,5'-cycloAMP in the present ELISA assay is approximately 2 nM. However, because the sensitivity of enzyme immunoassays is dependent on the signal-to-noise ratio (Chapter 2: INTRODUCTION), the conventional ELISA developed herein may have the potential to gain a higher signal-to-noise ratio through the use of

either a fluorogenic or radioactive enzyme substrate (see Chapter 2: INTRODUCTION). Both avenues shall be explored in subsequent attempts to increase the sensitivity of the current ELISA.

These studies shall be extended to detect formation of 8,5'-cyclo-AMP in irradiated polymers - initially polyadenylic acid, then to nucleic acids. The specificity and sensitivity of the antisera must be re-evaluated for the polymers since a lower yield of 8,5'-cycloAMP is anticipated due to competition of other nucleic acid constituents for hydroxyl radicals. In this context, the assay may be further enhanced by either an acid hydrolysis, which has the potential to cleave the C(1') - N(9') glycosidic bond thereby eliminating all the bases except the 8,5'-cycloAMP species, or an enzymatic hydrolysis which can perform a similar base cleavage, or a selective removal of one diastereoisomer, such as the (R) epimer by 5'-nucleotidase.

The ultimate performance of the ELISA is dependent upon the quality of the antiserum. Thus, because polyclonal antisera possess such a great degree of antibody heterogeneity, (see Chapter 2: INTRODUCTION) production of monoclonal antibodies to 8,5'-cycloAMP may be necessary to achieve the sensitivity necessary for radiobiological studies. This line of investigation is currently being pursued.

CONCLUSION

The radiation-induced intramolecular cyclization of 5'-AMP occurring in the absence of oxygen has been previously shown to lead to the formation of 8,5'-cycloAMP (Keck, 1968; Raleigh et al., 1970). This line of investigation raises the possibility that intramolecular cyclization may occur in irradiated nucleic acids. As a first step to ultimately assay for this product in nucleic acids, an enzyme-linked immunosorbent assay (ELISA) was developed using polyclonal sera raised to the 8,5'-cycloAMP moiety.

The radiation chemistry of 5'-AMP has been investigated in an attempt to characterize the products that might result during irradiation of polynucleotides. Irradiation of 5'-AMP under nitrous oxide leads to the formation of (R) and (S) 8,5'-cycloAMP. Intramolecular cyclization initiated by hydroxyl radical attack at the C(5') position of the ribose ring followed by subsequent addition to the C(8) position of the adenine base is clearly dependent on pKa-determined structural changes in 5'-AMP as initially proposed for formation of (S) 8,5'-cycloAMP (Raleigh et al., 1970). An acid stable material, which is tentatively attributed to hydroxyl radical attack at the C(8) position of the adenine base resulting in the formation of 8-hydroxyAMP, is correlated with decreasing susceptibility of C(5') to hydroxyl radical attack. Unequivocal identification of this product was not established at the time of writing. Therefore, from radiation chemical studies, formation of 8,5'-cycloAMP in irradiated nucleic acids may be dependent upon favorable conditions in terms of the protonation of the adenine base as

well as the ability to orient, unhindered, to a conformation favoring intramolecular cyclization.

Immunoassays are inherently specific in their ability to recognize antigenic determinants. Immunization of rabbits with a BSA-(S) 8,5'-cycloAdo conjugate prepared by the periodate-oxidation method of Erlanger and Beiser (1964) failed to form specific antibodies of the desired specificity. Two reasons are postulated for this failure: 1) cleavage of the C(2') to C(3') bond of the ribose ring may have resulted in a relaxation of the heterocycle ring as defined by C(1')-O-C(4')-C(5')-C(8)-N(9) and therefore, the antigenic determinants would be considerably different than the parent compound, or 2) the antibody binding sites with the greatest avidity may recognize determinants consisting of both the hapten and protein. An immunoassay was successfully developed from a conjugate formed by the covalent binding of 8,5'-cycloAMP through the terminal 5' phosphate to a protein carrier. In this preparation, the conformation of the hapten covalently bound to the protein is not perturbed and therefore, the aforementioned heterocyclic ring is maintained.

Enzyme-linked immunosorbent assays (ELISA), by virtue of the enzymatic degradation of a substrate as a means to assay the extent of the immunological reaction, have the potential to be amplified by a variety of means designed to increase the signal-to-noise ratio. Therefore, ELISA techniques are most appropriate to study radiation-induced modifications because of their potentially high sensitivities. The work presented herein demonstrates that the lower limit of detectability of 8,5'-cycloAMP in a conventional ELISA is approximately 1×10^{-9} M in the presence of 1.0 μM 5'-AMP, which is sufficient to detect 8,5'-cycloAMP formation at 330 Gy.

Enzyme-linked immunosorbent assays are inherently specific and have the potential to achieve high sensitivities. The question remains whether radiation-induced intramolecular cyclization occurs in irradiated polynucleotides and nucleic acids in the concentration range where immunochemical detection is feasible.

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APPENDIX A

FRICKE DOSIMETRY: CALCULATION OF THE ABSORBED DOSE OF CO-60 GAMMA RADIATION.

As reviewed by Fricke and Hart (1966) the absorbed dose may be calculated from:

$$D(\text{rads}) = \frac{N[\Delta(OD)]100}{(\Delta\varepsilon)10^{3}G(Fe^{3+})f\rho l[1 + C(t_2 - t_1)]}$$

where

- N = 0.023×10^{23} molecules/mole
- $\Delta(OD)$ = difference in optical density between irradiated solution and control
- $\Delta\varepsilon$ = difference in molar extinction coefficients ($M^{-1} \cdot cm^{-1}$) between ferric and ferrous ions, at the wavelength used for the optical density measurement
- ρ = density of irradiated solution = 1.024 for 0.8 N H_2SO_4
- l = optical path length (cm)
- f = 0.24×10^{18} eV/rad
- C = temperature coefficient
- t_1 = temperature at which ε was determined
- t_2 = temperature of solution from which OD was measured

The following parameters are constants: $\Delta\varepsilon$ @ 305 nm @ 25°C = 2174 litres/mole - cm; t_1 = 25°C; l = 1.0 cm; C = 0.7% /°C.

Therefore, the above equation simplifies to:

$$\text{Dose(rad)} = \frac{[\Delta(OD)] \times 0.05 \times 10^{2}}{2174(1.010 \times 10^{3})(15.6)[1 + 0.007(t_2 - 25)]}$$

$$\text{Dose(rad)} = \frac{[\Delta(OD)]}{2.80 \times 10^{4}[1 + 0.007(t_2 - 25)]}$$

$$\text{Dose(Gy)} = \frac{\text{Dose(rad)}}{\cdot 100}$$

APPENDIX B

PREPARATION OF ELISA BUFFERS

i) Coating Buffer (pH 9.6)

Na_2CO_3	1.50 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g
H_2O	1000 ml

ii) Phosphate Buffered Saline-Tween (PBS-T) (pH 7.4)

NaCl	8.0 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
KCl	0.2 g
Tween-20	0.5 ml
NaN_3	0.2 g
H_2O	1000 ml

iii) PBS-T (supplemented with 1% Goat Serum)

Goat Serum	0.2 ml
PBS-T	19.8 ml

iv) Goat anti-rabbit IgG Alkaline Phosphatase Conjugate
(1/400 dilution in PBS-T supplemented with 1% fetal calf serum)

FCS	100 µL
Conjugate	25 µl
PBS-T	9.0 ml

v) 10^{-2} Diethanolamine Buffer (pH 9.8)

Diethanolamine	97 ml
H_2O	800 ml
NaN_3	0.2 g

Adjust pH to 9.8 with 1.0 N HCl, then add H_2O to 1.0 L.

vi) Substrate

(Prepared immediately prior to use)

10^{-2} diethanolamine buffer	10 ml
Sigma 104™ Phosphatase Substrate	10 mg

vii) 3.0 N NaOH

NaOH	1.2 g
H_2O	10.0 ml

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CANADIAN THESES

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AN INVESTIGATION OF THE RADIATION CHEMISTRY AND THE DEVELOPMENT OF AN IMMUNOCHEMICAL ASSAY FOR 8,5' CYCLOADENOSINE-5' MONOPHOSPHATE

by

ALFRED ERNEST FOCTARELLI

A THESIS

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DEDICATION

For those who fill my life with joy and happiness

- my family and friends

To escape one's destiny

Is an exercise in futility.

-Alfred E. Fugiarelli

ABSTRACT

The radiation-induced intramolecular cyclization of 5'-AMP occurring in the absence of oxygen has been previously shown to lead to the formation of 8,5'-cycloAMP (Keck, 1968; Raleigh et al., 1970). This line of investigation raises the possibility that this chemical modification may occur in irradiated nucleic acids. However, detectability of this species is difficult because of the low yield of products anticipated from the irradiation of nucleic acids. Therefore, an assay for this type of chemical modification must be sensitive and specific.

Immunochemistry, by virtue of the interaction of antibodies with their antigenic determinants, has the potential to provide a high degree of specificity. Further, due to the amplification factor imposed by enzyme-catalyzed reactions, the enzyme-linked immunosorbent assay (ELISA) may provide the necessary sensitivity to detect 8,5'-cycloAMP in irradiated nucleic acids. Thus, an investigation of the applicability of ELISA methodology to probe for 8,5'-cycloAMP was initiated.

The radiation-induced modification of 5'-AMP was reinvestigated. Raleigh et al. (1970) and Raleigh and Blackburn (1978) initially demonstrated that the predominant product resulting from hydroxyl radical attack on C(5') of 5'-AMP was (S) 8,5'-cycloAMP for which the pH profile of formation is correlated with pKa-determined structure changes in 5'-AMP. The present work is in agreement with these findings but also illustrates that additional radiation-induced products are formed. One product has been identified as the (R) diastereoisomer of 8,5'-cycloAMP.

An enzyme-linked immunosorbent assay has been developed with specificity to 8,5'-cycloAMP. Polyclonal antisera were raised in rabbits by immunization with a conjugate prepared by covalently linking 8,5'-cycloAMP to a protein carrier through the 5'-terminal phosphate. Immunization of rabbits with a BSA-8,5'-cycloAMP conjugate formed by the periodate-oxidation method (Erlanger and Beiser, 1964) failed to generate antibodies with the desired specificity to the 8,5'-cycloAMP moiety. The ELISA assay has been applied to measure the formation of 8,5'-cycloAMP from irradiated solutions of 5'-AMP. 8,5'-CycloAMP may be measured in the presence of 2.3 μM 5'-AMP in free solution. With the present assay, 8,5'-cycloAMP may be detected in solutions of 5'-AMP irradiated to a total dose of $\geq$ 330 Gy.

This work shall form the basis for the development of an immunochemical assay to probe for 8,5'-cycloAMP in irradiated nucleic acids.

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ALFRED F. FUCIARELLI

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LIST OF SYMBOLS, NOMENCLATURE AND ABBREVIATIONS

CHEMICAL NAMES

8,5'-cycloAMP	8,5'-cycloadenosine-5'-monophosphate
8,5'-cycloAdo	8,5'-cycloadenosine
5'-AMP	adenosine-5'-monophosphate
5'-dAMP	deoxyadenosine-5'-monophosphate
3'-AMP	adenosine-3'-monophosphate
Ado	adenosine
dAdo	deoxyadenosine
Ade	adenine
5'-GMP	guanosine-5'-monophosphate
5'-dGMP	deoxyguanosine-5'-monophosphate
3'-GMP	guanosine-3'-monophosphate
Guo	guanosine
Gua	guanine
5-HMU	5-hydroxymethyluracil
5-HMUR	5-hydroxymethyluridine
5-HMUdR	5-hydroxymethyldeoxyuridine
t	thymine glycols (6-hydroxy or 6-hydroperoxy)-5,6-dihydrothymine
DNA	deoxyribonucleic acid
BSA	bovine serum albumin (Fraction V)
Im	imidazolide derivative
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline - Tween 20

EXPERIMENTAL AND THEORETICAL METHODS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
HRMS	High Resolution Mass Spectrometry
HPLC	High Pressure Liquid Chromatography
ELISA	Enzyme-linked Immunosorbent Assay
FL-ELISA	Fluorogenic Enzyme-Linked Immunosorbent Assay
USERIA	Ultrasensitive Enzyme Radioimmunoassay
RIA	Radioimmunoassay
UV VIS	Ultraviolet/Visible

SYMBOLS, ENTITIES AND UNITS

ppm	parts per million
G-value	molecules of product formed per 10 aJ (100 eV) absorbed
U	units
RT	retention time
ε	extinction coefficient ($M^{-1} \cdot cm^{-1}$)

MWCO	Molecular Weight Cutoff
mp	melting point
Hz	Hertz
n	molar ratio
rpm	revolutions per minute
IC_{50}	concentration (μM) of sample required for 50% inhibition
ID	Index of Dissimilarity
E_{410}	ELISA Value (410 nm)

CHAPTER 1: INTRODUCTION

Chromosomal DNA has been implicated as the major target for the lethal and mutagenic effects of ionizing radiation on living cells. The molecular changes responsible for the biological manifestations of ionizing radiation have not been clearly identified. Among the radiolytic lesions occurring in DNA are crosslinks (Charlesby, 1960; Bohne et al., 1970; Fornace and Little, 1977), strand breaks (see reviews by Ward, 1972; von Sonntag et al., 1981), base damage (see reviews by Téoule and Cadet, 1978) and sugar damage (see reviews by von Sonntag and Schulte-Frohlinde, 1978; von Sonntag et al., 1981).

Much of radiation chemistry is dependent on the selection of appropriate models to study the possible mechanism and significance of radiation-induced events which may occur when DNA is irradiated *in situ* in the cell (see Chapter 1). In an effort to address the underlying molecular events *in vivo*, monomeric nucleosides, nucleotides, nucleic acid bases and sugar moieties are the subject of many radiation chemical studies. However, the extrapolation of the results obtained with such monomeric species irradiated under various gassing and buffer environments to the radiation chemical behavior of nucleic acids *in situ* in the cell, must be done only with extreme caution.

Radiation-induced modifications to monomeric species are more easily assayed than similar modifications which may occur upon irradiation of DNA since the concentration of reactants can be greater and the number of products formed is more restricted in these model systems. On the other hand, direct identification and measurement of product yields from

irradiated DNA is complicated by three major factors: 1) the vast quantity of structurally similar chemical products resulting from radiolytic degradation of DNA requires an assay with exquisite specificity for an individual radiation product; 2) the low yield of radiation-induced chemical products necessitates an assay with very high sensitivity and 3) the radiation-induced products are expected to be of limited chemical stability and therefore, isolation procedures for measurement and characterization must be inherently mild and if possible, avoided.

In light of these factors, immunochemistry may provide a means for the detection of specific radiation-induced products formed in DNA. Immunochemistry offers the advantages of: 1) high specificity due to the inherent recognition of antigenic determinants by the antibody binding sites and 2) great sensitivity due to the advances in the development of inhibition assays. Optimization of both of these features has the potential to increase the detectability of products formed during the radiolytic decomposition of DNA as compared to conventional assays. Potentially limiting factors for this type of immunoassay are: 1) limitations on the quality of the assay as reflected by the avidity of the antibodies to the antigenic determinant and 2) product yields, which as a result of steric hindrance or scavenging of hydroxyl radicals by histone or non-histone proteins intercalated with DNA (Emmerson et al., 1960; Lloyd and Peacocke, 1965) might be below the lower limits of detectability.

Radiation-induced chemical lesions in DNA have been studied by a variety of analytical techniques which are summarized in Table 1. An

exhaustive literature listing pertaining to each method used to measure DNA damage has been avoided. Rather, a sampling of the types of assays to define and clarify each method has been presented.

TABLE I: METHODS USED TO INVESTIGATE RADIATION-INDUCED PRODUCTS IN DNA

Strand Break Analysis:

Kaplan, 1966
McGrath and Williams, 1966
Bohme et al., 1970
Sawada and Okada, 1970
Ormerod and Stevens, 1971
Karren and Ormerod, 1973
(a) Hüttermann et al., 1978

Enzymatic End Group Analysis:

Bopp and Hagen, 1970
Bopp et al., 1973
Lennartz et al., 1975
(a) Ward, 1975
(a) von Sonntag et al., 1981

Analytical Chemistry:

i) Chromophore destruction at 260 nm:

Blok et al., 1967
Ward and Kuo, 1970

ii) Products released upon irradiation without subsequent hydrolysis:

Hems, 1960a
Scholes et al., 1960
Simon, 1969
Kapp and Smith, 1970
Ullrich and Hagen, 1971
Dizdaroglu et al., 1975 a,b
Richmond and Zimbrick, 1975
Polverelli et al., 1976
Bonicel et al., 1980
Chetsanga and Grigorian, 1983

iii) Products released following hydrolysis of irradiated DNA:

a) acid/alkali hydrolysis at elevated temperatures:

Téoule et al., 1977
Dizdaroglu et al., 1977 a,b

Table 1 cont'd

b) enzymatic hydrolysis

Dizdaroglu et al., 1978

Damage-Specific Endonucleases:

Paterson and Setlow, 1972
Brent, 1973
Strinste and Wallace, 1975
Armel et al., 1977
Gates and Linn, 1977

Radiolabelled DNA Product Analysis:

i) Following hydrolysis:

Hariharan and Cerutti, 1972
Roti-Roti et al., 1974
Hariharan, 1980
Bonjeel et al., 1980

ii) Released under physiological conditions as a result of enzymatic excision processes:

Hariharan and Cerutti, 1972
Cerutti, 1974
Roti-Roti et al., 1974
Swinehart and Cerutti, 1975
Dunlap and Cerutti, 1975
Ward and Kuo, 1976

Immunochemical Detection of Radiolytic Products from DNA:

Lewis et al., 1978
Lewis and Ward, 1978
West et al., 1982 a
West et al., 1982 b
Leadon and Hanawalt, 1983
Rajagopalan et al., 1984

(a) Review articles

Desirable goals for an assay which is designed to detect specific radiation-induced chemical modifications in DNA are high specificity and sensitivity. Many of the assays listed in Table 1 are limited in their

ability to achieve these goals. Strand break analysis may be the ultimate result of a multitude of chemical alterations to DNA. Ionizing radiation induces not only direct scissions of the nucleotide chain, but also modifications in the sugar moiety which can subsequently lead to chain scission under alkaline conditions. This latter phenomenon is referred to as "alkali-induced strand breakage". The ability to identify a chemical modification and attribute that modification to a subsequent strand break is tentative. Similarly, enzymatic end group analysis of the strand breaks is limited. Identification of hydroxyl or phosphate end groups on the 3'- or 5'-terminals is facilitated by the large number of enzymes which act on these termini. However, enzymes which use other substrates have recently been reported (Janicek et al., 1984). The mechanism leading to the formation of these products is equally complicated since base destruction, nucleotide liberation, or sugar damage can result in strand breaks with hydroxyl or phosphate termini. The use of damage-specific endonucleases to nick DNA, may be a sensitive measure of radiation-induced chemical lesions, but the identity of such lesions may be difficult to establish.

The analysis of total base destruction resulting from ionizing radiation in DNA has been achieved by analytical chemistry techniques. However, due to the chemical nature of DNA, many of these techniques are limited. The loss of ultraviolet absorption of DNA in the 260 nm region has been observed as a function of dose after denaturation of the irradiated polymer. Underlying the use of this assay is the premise that the addition of water radiolysis products to the purine and pyrimidine bases leads to a loss of ring aromaticity. However, these assays are limited to the destruction of ring aromaticity and fail to measure, for

example, the anoxic products of the radiolytic decomposition of 5'-AMP (see Chapter 1) in which the glycosidic bond is not cleaved. Further, the use of optical absorbance measurements for the assay of base destruction in DNA is dependent on the assumption that the major reactions occurring in monomers also occur in the polymers - an assumption that may not be justified. Radiation-induced changes in hyperchromicity, resulting from hydrogen bond breakage and local denaturation of the polymer, represent a serious additional complication for the use of ultraviolet spectroscopy in measuring base damage.

Hydrolysis of the DNA polymer prior to product analysis with: 1) strong acids and bases at elevated temperature or 2) enzymes under physiological conditions has introduced other complications to assays. Acid hydrolysis may possibly modify a portion of the radiation products or induce their reversion back to the starting materials. Furthermore, acid-labile products and products resistant to acid hydrolysis, such as 8,5'-cycloAMP (Chapter 1), may not be detected under some conditions. Endonucleases, which degrade polydeoxyribonucleotides stepwise starting at the free 3'- or 5'-hydroxyl termini, yielding 3'- or 5'-nucleoside monophosphates, have provided useful information regarding base damage in irradiated DNA. However, complete hydrolysis is not always achieved (Dizdaroglu et al., 1978) and this may complicate subsequent analysis.

The development of a reductive assay to detect radiolytic damage of thymine bases by Hariharan and Cerutti (1971, 1972) represented a major step forward in the identification of specific chemical lesions in DNA polymers. The assay is specific for the major aerobic radiolysis products of thymine, which are thymine glycols (t^) [6-(hydroxy or hydroperoxy)-5,6-dihydrothymine] and their hydantoin analogues [5-(hydroxy or hydroperoxy)-5-methylhydantoin]. These products are expected to be reduc-

tively cleaved by $NaBH_4$ to form a urea derivative together with 2-methyl-glycerol, 1,3-dihydroxy-2-methylpropane and possibly 1,2-dihydroxypropane from the hydantoin analogues (Hariharan and Cerutti, 1971): (Figure 1). Alternatively, the saturated ring of the radiation products can also be fragmented by consecutive treatment with alkali and acid (Hariharan and Cerutti, 1974) or with alkali alone (Hariharan, 1980) yielding acetol and carbon dioxide.

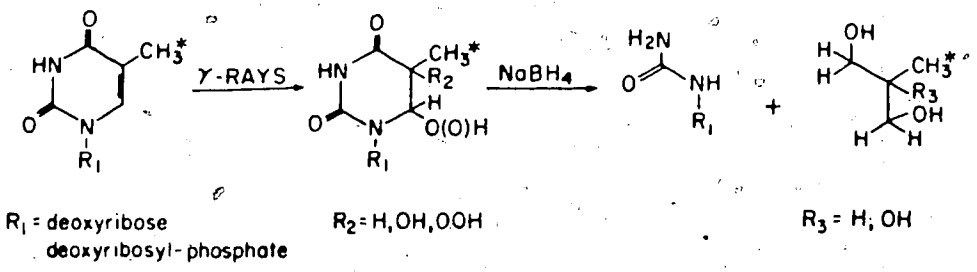

FIGURE 1: Schematic diagram of the principal steps involved in the reductive assay for the radiolysis of thymine (From Hariharan and Cerutti, 1972).

The formation of two types of radiation-induced thymine damage in DNA *in vitro* and *in vivo* can be assayed with these techniques by measuring either the gamma-ray-induced production of $[^3H]H_2O$ from [methyl-^{3}H] thymine and/or the formation of thymine glycol derivatives. The selectivity of the reductive assay is due to the specificity of $NaBH_4$ reduction with thymine glycols formed in irradiated DNA. However, the

reductive assay has the following limitations: 1) there are a number of structurally similar radiation products of thymine, consisting of thymine glycols and hydantoin analogues, which are assayed as one type of damage immediately following irradiation or subsequent to a post-irradiation incubation which prevents assignment of yields of individual chemical species and 2) high radioactivity backgrounds are obtained when the assay is applied to DNA isolated from cells grown in the presence of [methyl-^{3}H] thymine of which the origin is unknown. Despite these limitations, the reductive assay has been used to detect and follow the rate of removal of thymine glycol from DNA following irradiation of Micrococcus radiodurans (Hariharan and Cerutti, 1972), Chinese hamster ovary cells (Mattern et al., 1973), isolated nuclei from Hela cells and human lung fibroblasts (Remsen et al., 1974) and Escherichia coli lysates (Hariharan and Cerutti, 1974). By application of the reductive assay, Swinehart and Cerutti (1975) demonstrated a linear relationship between the loss of biological activity and the formation of thymine glycols in ϕ X 174 bacteriophage and E. coli cells.

Application of immunochemical techniques to assay radiation-induced chemical lesions in DNA offers attractive advantages in terms of assay specificity and sensitivity over other techniques applied in radiation chemist: . Immu[illegible]says may be designed to recognize radiolytic damage in DNA with[illegible]he need for extensive hydrolysis or the need for radioactive labelling of unknown products. However, both of these techniques may be incorporated into immunochemical protocols if so desired. With the advent of radioimmunoassays (RIAs), sensitivity of immunoassays was increased to the point where picomoles of material could be assayed. Similar

sensitivity without the use of radiotracers has become possible with the introduction of enzyme-linked immunosorbent assays (ELISA) (Engvall and Perlmann, 1971; 1972; Engvall et al., 1971). The sensitivity of enzyme immunoassays has been increased by recent techniques such as the use of fluorogenic substrates for enzyme analysis (FL-ELISA) (Shalev et al., 1980) and the introduction of a radioactive substrate for enzyme quantification (RS-ELISA) (Harris et al., 1979). This increased sensitivity may be necessary to achieve detectability of radiolytic products formed in DNA.

Lewis et al. (1978) developed a phage neutralization assay with specificity to 5-hydroxymethyldeoxyuridine (5-HMUdR), a product formed in irradiated thymidine solutions. The antibodies were raised against a BSA-5-hydroxymethyluridine conjugate and the prime determinant was found to be the 5-hydroxymethyluracil (5-HMU) moiety. The limit of detectability of 5-HMUdR in a neutral aqueous solution of thymidine saturated with oxygen was 2×10^{-7} M (Lewis and Ward, 1978) with a corresponding G(5-HMUdR) = 0.05 (Lewis et al., 1978; Lewis and Ward, 1978). The yield of 5-HMU in oxygen-saturated irradiated solutions of DNA was G(5-HMU) = 0.05. Therefore, the 5-HMUdR moiety may be detected at levels of one nucleotide per 3×10^{6} daltons of DNA, with the lower limit of detection of product formation at 1.0 kGy (Lewis et al., 1978; Lewis and Ward, 1978).

Immunochemical analysis of radiation-induced damage to adenine-based nucleic acid constituents has been investigated. In a preliminary report by Lewis and Ward (1978), a phage neutralization assay was developed with specificity to 8,5'-cycloadenosine which was supplied as the (S)

diastereoisomer by Raleigh et al. (1970). Under aerobic conditions no cycloAdo was formed in irradiated solutions of 5'-AMP, but the yield from irradiated deoxyadenosine (dAdo) was reported to be G 0.00. The formation of 8,5'-cycloAdo was also detected in nitrogen-saturated solutions of dAdo and Ado with yields of G 2.30 and G 0.043 respectively (Lewis and Ward, 1978). However, the yield of cycloAdo from irradiated solutions of dAdo by these authors is disturbingly high considering that the yield of hydroxyl radicals from water radiolysis is G 2.7 (Scholes, 1983) and that hydroxyl radicals are primarily responsible for the intramolecular cyclization (Raleigh et al. 1970). West et al. (1982b) developed a radioimmunoassay which could detect 4 x 10^{-14} moles of 8-hydroxyadenosine. Formation of 8-hydroxyadenosine could be detected at 10 Gy and the yield of this product from DNA, irradiated under aerobic conditions, was reported as G 0.12 (West et al., 1982b). By comparison, Bonicel et al. (1980) required 500 - 4000 Gy to liberate a detectable quantity of 8-hydroxyadenosine from irradiated DNA using analytical chemical techniques.

Radioimmunoassays for cis-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) have been developed (West et al., 1982a; Teadon and Hanawalt, 1983; Rajagopalan et al., 1984). As little as 4 femtomoles of thymine glycol have been detected in OsO_4 treated DNA (West et al., 1982a). This same assay can detect thymine glycol in gamma-irradiated DNA at a G-value of 0.018. Detection of this product may occur following 200 Gy of gamma-radiation (West et al., 1982a; Rajagopalan et al., 1984) which has comparable sensitivity to the alkali-degradation assay (Hariharan and Cerutti, 1972) which measures a yield of G(t) 0.06. Since the alkali-degradation assay measures all hydroxy-hydroperoxy and dihydroxy

analogues, the higher G-value may represent the lack of specificity inherent in the alkali-degradation assay. Leadon and Hanawalt (1983) developed monoclonal antibodies which could detect one thymine glycol in 2.2×10^5 thymines. The assay detected thymine glycol formation in irradiated solutions of DNA with doses as low as 2.5 Gy.

Application of immunochemistry to the field of radiation chemistry requires selection of an appropriate chemical lesion which may occur upon irradiation of DNA in situ in the cellular environment. As probes of radiation-induced damage in DNA, 8,5'-cyclonucleotides (Chapter 1) are attractive for a number of reasons: 1) this type of chemical lesion represents an intramolecular transfer of radiation damage from the sugar-phosphate moiety to the nucleotide base. The formation of this type of product may be important in double-stranded nucleic acids where the sugar phosphate backbone is exposed to, and the bases are shielded from, direct radical attack; 2) 8,5'-cycloadenosine-5'-monophosphate is extremely stable to acid hydrolysis; 3) 8,5'-cyclonucleotides can be covalently linked to proteins to form immunogens (Chapter 2) and 4) 8,5'-cycloAMP does not act as a substrate for certain enzymes and therefore raises speculation as to the ability of repair enzymes to recognize and rectify such radiation-induced chemical modifications (Chapter 1). This latter point ultimately raises questions concerning the biochemical significance of such modifications if they are indeed a result of irradiation of DNA in the cellular environment.

In an effort to investigate the potential applicability of immunochemical assays as probes for specific radiation-induced lesions in DNA irradiated in situ in the cellular environment, 8,5'-cycloAMP was

investigated as a model for nucleotide damage. Development of this project proceeded along two avenues of investigation which resulted in the presentation of: 1) a model in which the radiolytic degradation of adenosine-5'-monophosphate under nitrous oxide saturation led to the formation of both diastereoisomers of 8,5'-cycloadenosine-5'-monophosphate (Chapter 1) and 2) an immunochemical assay which was both specific and sensitive for the detection of this product in irradiated solutions of 5'-AMP (Chapter 2).

CHAPTER 2:

RADIATION CHEMISTRY OF ADENOSINE-5'-MONOPHOSPHATE: A MODEL SYSTEM FOR INTRAMOLECULAR TRANSFER OF RADIATION DAMAGE

INTRODUCTION

Radiation-induced chemical alterations in the structure of deoxyribonucleic acid (DNA) and the biochemical manifestations resulting from these events in the living organism, are largely related to water radiolysis. Biomolecules can undergo radiation damage by two possible mechanisms: 1) direct ionization and excitation ("Direct Effect") and 2) chemical changes resulting from the absorption of energy by water molecules leading to the formation of reactive intermediates which, in turn, interact with biomolecules ("Indirect Effect"). As a result of the high concentration of water in living tissue, most radiation damage to biomolecules occurs as a result of chemical modification via the indirect effect.

The radiation-induced decomposition of water has been extensively studied (recent review: Schwarz, 1981) and for the purposes of this discussion may be briefly summarized as in Table 1. Absorption of ionizing radiation by water molecules leads to the formation of three short-lived reactive species - the hydrated electron (e^-_{aq}), the hydrogen atom and the hydroxyl radical within the spur (Mozumder and Magee, 1966). The proposed mechanisms of formation of these species and their final yields, given in terms of their G-values [viz., molecules of product produced per 10 aJ (100 eV) absorbed], are indicated in Table 1. As these radicals diffuse outward from the spur, recombination reactions occur leading to the formation of the molecular products - hydrogen peroxide and molecular hydrogen.

In dilute, aqueous solutions of nucleosides, nucleotides and DNA, the efficiency of attack by species emanating from water radiolysis are assessed by reaction rate constants as elucidated from pulse radiolysis and other kinetic studies (see Scholes, 1970). Reactions of hydrated electrons with the free bases are extremely rapid indicating that these

TABLE 2: ACTION OF IONIZING RADIATIONS ON WATER

$$H_2O \rightsquigarrow e^-_{aq},\ H^\cdot,\ {}^\cdot OH,\ H_3O^+,\ H_2,\ H_2O_2$$

Radical products

$$H_2O \rightsquigarrow H_2O^+ + e^-$$

$$e^- + nH_2O \longrightarrow e^-_{aq}$$

$$H_2O^+ + H_2O \longrightarrow {}^\cdot OH + H_3O^+$$

$$H_2O \longrightarrow H_2O^\cdot \longrightarrow H^\cdot + {}^\cdot OH$$

$$e^-_{aq} + H_3O^+ \longrightarrow H^\cdot + H_2O$$

Molecular products

$$H^\cdot + H^\cdot \longrightarrow H_2$$

$$e^-_{aq} + e^-_{aq} \xrightarrow{2H_2O} H_2 + 2OH^-$$

$$e^-_{aq} + H^\cdot \xrightarrow{H_2O} H_2 + OH^-$$

$${}^\cdot OH + {}^\cdot OH \longrightarrow H_2O_2$$

Yields

Species:	e^-_{aq}	$H^\cdot$	${}^\cdot OH$	H_3O^+	H_2	H_2O_2
G:	2.7	0.55	2.7	2.7	0.45	0.7

[G = molecules per 100 eV; 1 eV = 0.16 aJ]

(Adapted from Scholes, 1983)

reactions are diffusion controlled. The lack of affinity of the sugar for the hydrated electron implies that attack by this reducing species on the polynucleotide will occur exclusively on the base components. Hydroxyl radicals, which are the oxidizing species, react with both base and sugar components. Estimates of attack of hydroxyl radicals on the sugar moiety range from 10% - 20% (Scholes et al., 1960; Scholes et al., 1969; Jung et al., 1969; Hoard et al., 1974).

Products formed during the radiolytic decomposition of water have been demonstrated to result in significant amounts of damage in vitro. A correlation between the ability of various compounds to scavenge radicals resulting from water radiolysis, and the radioprotective effect of such compounds was observed when these compounds were present during irradiation of E. coli B/r (Johansen and Howard-Flanders, 1965; Sanner and Phil, 1960). The radioprotective ability of these compounds correlated well with their calculated rate constants for reaction with hydroxyl radicals. However, no correlation was found between the radioprotective ability and the rate constant for either the hydrated electron or the hydrogen atom. Chapman et al. (1973) demonstrated with the radioprotector dimethyl sulfoxide that approximately 62% of the radiation inactivation observed in air-saturated Chinese hamster fibroblasts is a direct consequence of hydroxyl radical attack resulting from water radiolysis. Roots and Okada (1972) demonstrated a similar correlation between the radioprotective ability and the efficiency by which the compound could scavenge hydroxyl radicals as measured by a decrease in the strand break yield from the DNA of mouse L5178 cells. These experiments illustrate that hydroxyl radical induced damage in cells can be lethal and that hydroxyl radicals induce

single strand breaks in cellular DNA, but do not demonstrate a clear relationship between these two observations.

Roots (1970) has calculated that hydroxyl radicals may migrate a distance of 4.0 nm from their site of formation. Thus, a significant proportion of the hydroxyl radical-induced damage to DNA must result from radicals formed in the hydration layer surrounding the macromolecule. Michaels and Hunt (1973) have calculated that the reaction rate constant of hydroxyl radicals with DNA is 5×10^8 $M^{-1}.s^{-1}$ per base using the Braams and Ebert (1968) relationship. The observed rate constant of 4×10^8 $M^{-1}.s^{-1}$ per base suggests that hydrogen abstraction from the sugar moiety in DNA may be more significant than previously thought (Scholes et al., 1960; Scholes et al., 1969; Jung et al., 1969; Hoard et al., 1974; Ward, 1975; von Sonntag and Schulte-Frohlinde, 1978). Radiation-induced base destruction is greater for pyrimidine bases, deoxynucleosides and deoxynucleotides than for the corresponding purine constituents (Ward, 1975). The yield of adenine destruction from polyadenylic acid irradiated as a random coil was equivalent to the yield of adenine destruction when the free base was irradiated [G(-adenine) = 0.65] (Ward and Urist, 1967). However, the yield of base destruction for polyadenylic acid irradiated in the double helical form was significantly lower [G(-adenine) = 0.34], suggesting that the bases are sterically protected within the double helical matrix from radical attack. Ward and Kuo (1970) have demonstrated a lag in adenine destruction at low doses upon irradiation of polyA and DNA in the double helical form. Ward and Kuo (1973) demonstrated that the shoulder of the base destruction curve is reduced from 200 Gy to 100 Gy if 0.7% of the phosphodiester bonds are removed by "nicking" the DNA

helix with DNase prior to irradiation. Complete removal of the shoulder occurs after 1.7% of the phosphodiester bonds are removed (Ward and Kuo, 1973). The shielded base hypothesis is further supported by pulse radiolysis studies in which a reduction in the optical absorption signal of radiation-induced DNA-OH$^{\cdot}$ radicals is observed, as compared to that expected for a mixture of deoxynucleotides. The intensity of the absorption signal increases if the DNA is preirradiated (Scholes et al., 1960). Experiments utilizing other techniques to assess DNA damage have reported similar shoulders in the dose-yield plots (c.f. Ward, 1975).

Successive reactions of the radicals formed on DNA will be affected by the relative immobility imposed on them due to their presence in the macromolecule and are made possible by their long lifetime. Subsequent reactions of the DNA radical may include: 1) removal of the radical by smaller diffusing radicals (or possibly electron transfer down the DNA chain); 2) unimolecular decay of the macroradical; 3) reaction with small molecular species and 4) intramolecular or intermolecular reactions. Recent reviews which discuss chemical modifications to the constituents of the nucleic acids including base damage (Téoule and Cadet, 1978), sugar damage (von Sonntag and Schulte-Frohlinde, 1978) and nucleotide or nucleoside damage (Scholes, 1978; von Sonntag et al., 1981) present a much broader survey of the radiation chemistry than shall be presented here. However, for the purpose of discussion relevant to the possible radiation-induced chemical alterations occurring in adenosine-5'-monophosphate, a brief review of selected literature shall be presented.

The radiation-induced degradation of aqueous purine nucleosides and nucleotides leads to three types of reactions:

1) Cleavage of the N-glycosidic bond followed by liberation of the base and cleavage of the O(5')-P bond resulting in phosphate release (Scholes and Weiss, 1952; Daniels et al., 1956).
2) Change in structure of the base and sugar components without rupture of the N-glycosidic bond (Hems, 1958, 1960b,c).
3) Intramolecular cyclization as demonstrated by the formation of 8,5'-cyclonucleotides (Keck, 1968; Raleigh et al., 1970).

These reactions parallel chemical alterations observed in irradiated DNA (see INTRODUCTION) and therefore, the selection of 5'-AMP as a model to study radiation-induced DNA damage appears appropriate.

The radiolytic decomposition of adenine suggests mechanistic patterns which may be relevant to nucleotide decomposition (Figure 2). The major species resulting from irradiation of adenine in aqueous solutions under both aerobic and anoxic conditions is 8-hydroxyadenine which exists as a mixture of enol(Ia) and keto(Ib) tautomers (Conlay, 1963; Ponnamperuma et al., 1963; van Hemmen and Bleichrodt, 1971). Formation of this species provides evidence for hydroxyl radical attack at the N(7) - C(8) position of the imidazole ring which confirms quantum chemical calculations of adenine spin densities (Pullman and Pullman, 1963; Müller and Hüttermann, 1973). Further radiolytic degradation of this product resulting from ring fragmentation of the imidazole ring, would account for the formation of minor secondary species (Mariaggi and Téoule, 1974). Hypoxanthine, 6-amino-8-hydroxy-7,8-dihydropurine (II) and 4,6-diamino-5-formamido-pyrimidine (III) have also been identified in

anoxic solutions (Ponnamperuma et al., 1961, 1963; van Hemmen and Bleichrodt, 1971). Reconstitution reactions involving adenine radicals in anoxic solutions prevent significant degradation of adenine (Conlay, 1963; van Hemmen and Bleichrodt, 1971).

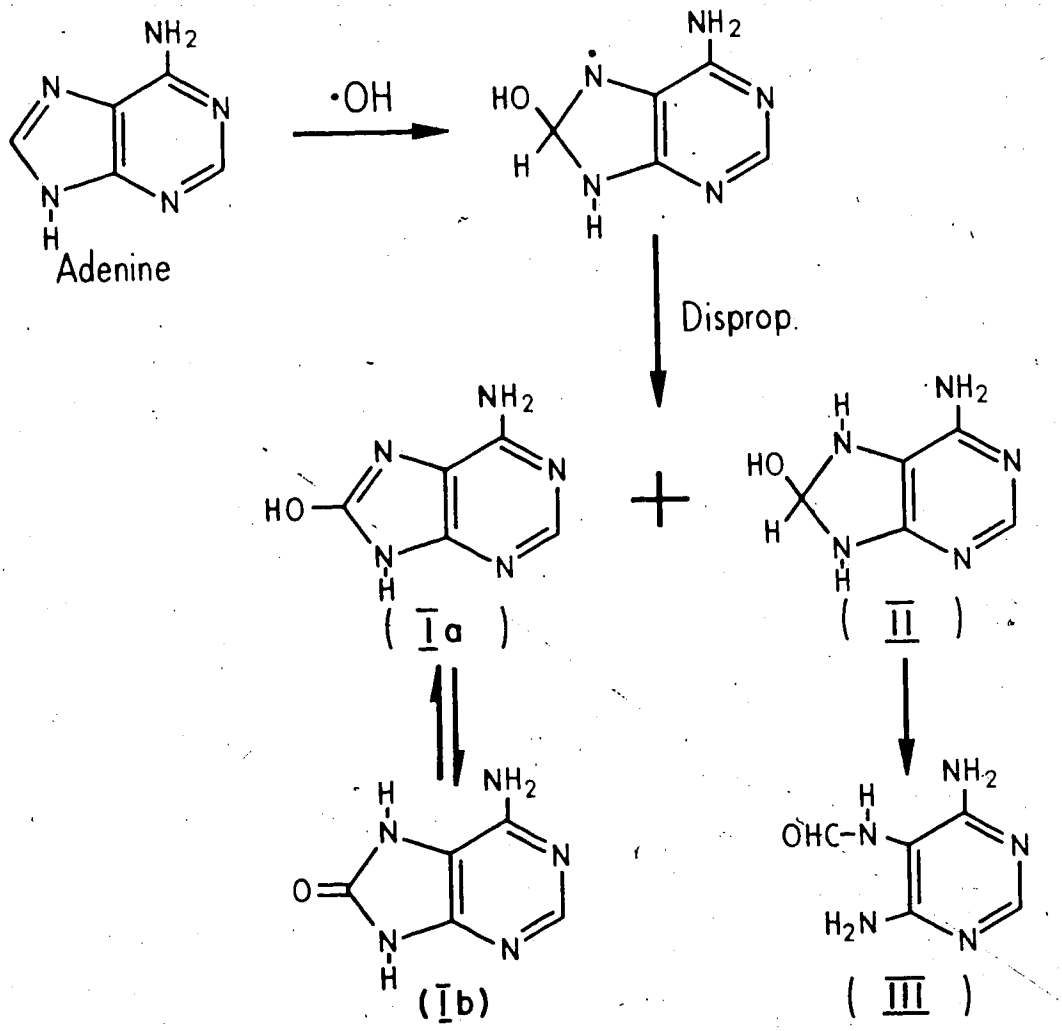

FIGURE 2: Schematic diagram of the mechanism for the radiolytic decomposition of adenine under aerobic and anaerobic conditions.

The possible modes of radiolytic degradation of 5'-AMP are partially summarized in Figure 3. Keck (1968) demonstrated by ^{1}H-NMR spectroscopy that an intramolecular cyclization involving the formation of a C(5') to C(8) bond and resulting in 8,5'-cycloadenosine-5'-monophosphate occurred upon irradiation of a nitrogen-saturated solution of 5'-AMP. The mechanism leading to the formation of such cyclonucleotides is initiated by hydroxyl radical abstraction of a hydrogen atom from the C(5') carbon of the ribose ring (I). Cyclization is completed by radical substitution at the C(8) carbon of the purine (II) - a center which has proven to be highly reactive (Pullman and Pullman, 1963) and where substitution by various alkyl radicals has been established (Leonov and Elad, 1974; Zady and Wong, 1980; Shetlar et al., 1980). A subsequent one-electron oxidation produces the cyclonucleotide (III). Keck (1968) postulated that hydroxyl radical attack leads to the formation of Radical (IV) which, in turn, leads to 6-amino-8-hydroxy-7,8-dihydroadenosine-5'-monophosphate (V), which could undergo further hydrolytic degradation to the substituted pyrimidine (VI). However, by analogy with the radiolytic degradation of adenine (Ponnamperuma et al., 1961, 1963; van Hemmen and Bleichrodt) and 2'-dAMP (Mariaggi and Téoule, 1976) the possible formation of 8-hydroxyadenosine-5'-monophosphate (VIIa,b) from Radical (IV) may also be anticipated. Possible species resulting from the radiolytic degradation of 5'-AMP that are not illustrated include adenine - resulting from hydroxyl radical attack at C(1') and hypoxanthine derivatives.

Radiation-induced alterations to the sugar moiety on 5'-AMP may also be anticipated. Mariaggi et al. (1979) have suggested a radiolysis

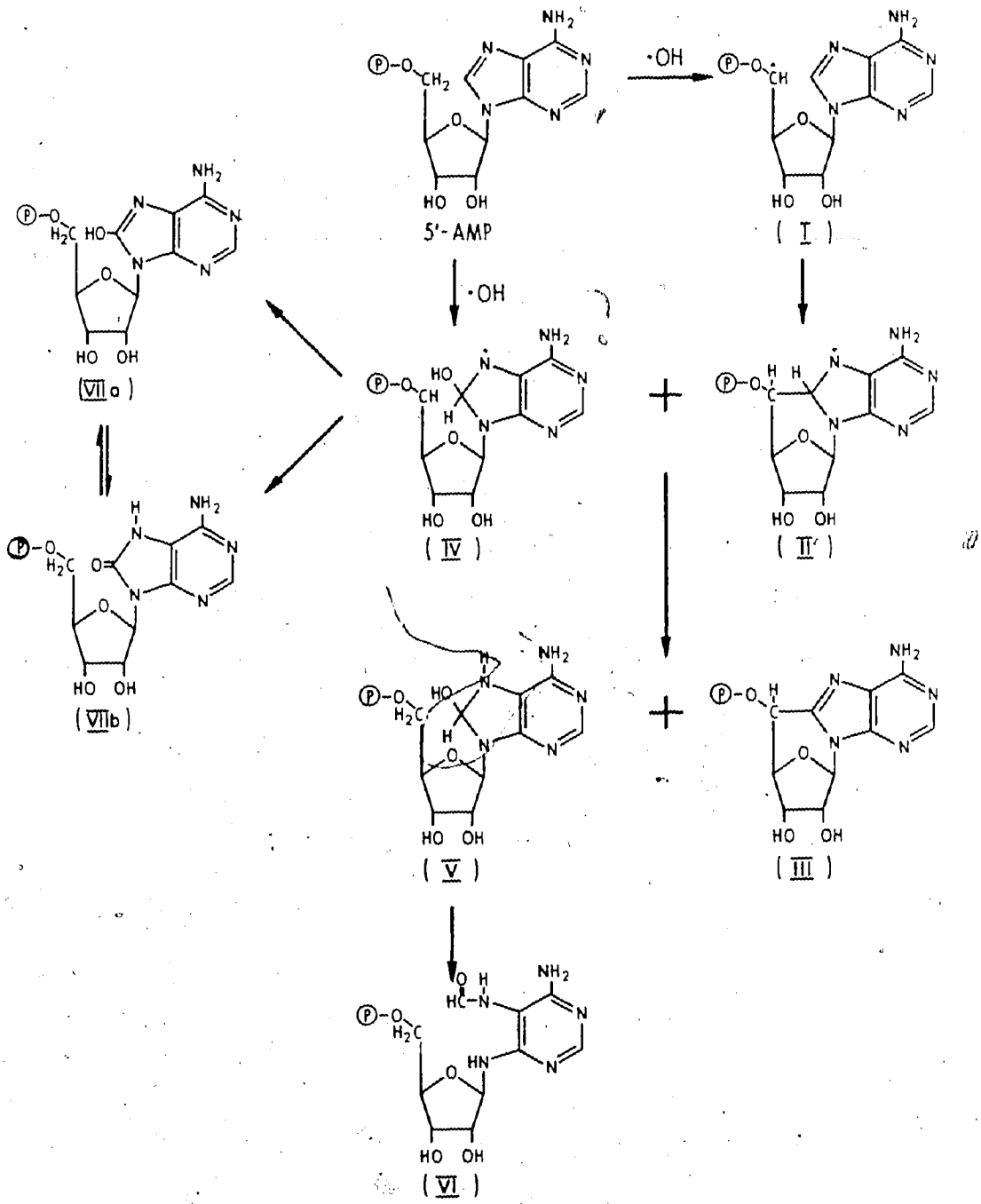

FIGURE 3: Schematic diagram of the mechanism for the radiolytic decomposition of adenosine 5'-monophosphate under anaerobic conditions.

mechanism consistent with the formation of 9-(2-deoxy- α-D erythropentopyranosyl) adenine (I), 9-(2-deoxy- β -D-erythropentopyranosyl)adenine (II), and 9-(2-deoxy- α -D-erythropentofuranosyl)adenine (III) from irradiated solutions of 2'-deoxyadenosine saturated with argon gas (Figure 4). The formation of these compounds may result from cleavage of the C(1')-O bond of the deoxyribose residue during radiolysis which subsequently leads to rearrangement of the initial molecule into its four α - and β -pyranoic and furanoic forms (Mariaggi et al., 1970). In contrast to the mechanism proposed for 8,5'-cycloAMP formation, this mechanism is dependent on hydrogen abstraction by hydroxyl radicals from the C(4') carbon (Dizdaroglu et al., 1975b; Hartmann et al., 1970).

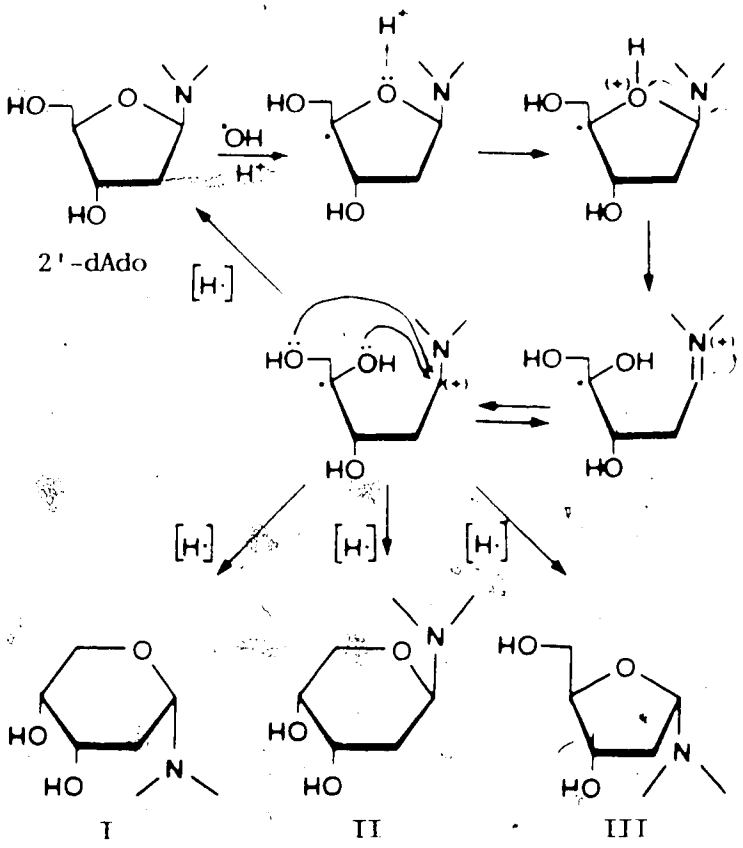

FIGURE 4: Schematic diagram of the mechanism for the radiolytic decomposition of 2'-deoxyadenosine (From Mariaggi et al., 1970).

As discussed above, Keck (1968) characterized the structure of 8,5'-cycloadenosine-5'-monophosphate by demonstrating the absence of the ^{1}H-NMR resonance peak which would have been contributed from the C(8) proton of the adenine ring in 5'-AMP. This report failed to indicate the nature of the diastereoisomer(s) formed at the C(5') position. Organic synthesis of epimeric mixtures of the (R) and (S) diastereoisomers has been achieved by ultraviolet photolysis of 5'-substituted adenosine derivatives (Hogenkamp, 1963; Harper and Hampton, 1972; Hampton et al., 1972). Matsuda et al. (1978) have achieved organic chemical synthesis of both diastereoisomers of 8,5'-cycloadenosine via the 2',3'-O-isopropylidene derivatives which can be converted to the corresponding 5'-phosphates by the cyanoethyl-phosphate method (Tener, 1961). Radiation chemical synthesis of 8,5'-cycloAMP is also available and while the yield is modest, the synthesis is technically simple, requiring only a preparative anion exchange chromatography for product isolation.

The mechanism of formation of 8,5'-cycloAMP during irradiation of 5'-AMP in anoxic solutions has been studied by Raleigh et al. (1976). Radical formation resulting from hydroxyl radical attack has been demonstrated by: 1) comparison of the yield of 8,5'-cycloAMP irradiated under nitrous oxide or nitrogen saturation and 2) a decreasing yield of 8,5'-cycloAMP in the presence of increasing yields of the hydroxyl radical scavenger t-butyl alcohol (Raleigh et al., 1976). Since nitrous oxide converts hydrated electrons to hydroxyl radicals by Reaction 1 (Dainton and Peterson, 1962; Dainton and Watt, 1963),

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + {}^{\cdot}OH + OH^- \qquad (1)$$

$$k = 5.6 \times 10^9\ M^{-1}.s^{-1}$$

an effective doubling of the steady state concentration of hydroxyl radicals occurs in this environment. The nitrous oxide experiment in which hydrated electrons are converted to hydroxyl radicals (Reaction 1) rather than to hydrogen atoms, indicates that hydrogen atoms contribute little to the formation of 8,5'-cycloAMP as the yield of 8,5'-cycloAMP is enhanced by a factor of 2.0 at pH 3.0 in the presence of N_2O (Raleigh et al., 1970). Therefore, the increased yield of 8,5'-cycloAMP under N_2O saturation, as compared to N_2 saturation at pH 3 wherein e^-_{aq} is converted to H$^\cdot$ ($e^-_{aq} \cdot H^+ \longrightarrow H^\cdot$, $k = 2.30 \times 10^{10}\ M^{-1} \cdot s^{-1}$) demonstrates that the hydroxyl radical is the major chemical species governing the formation of 8,5'-cycloAMP.

The yield of 8,5'-cycloAMP, later reported as the (S) diastereoisomer at the C(5') carbon by ^{1}H-NMR spectroscopy (Raleigh and Blackburn, 1978) and X-ray crystallography (Haromy et al., 1980) was studied as a function of the pH of the solution (Raleigh et al., 1970). A close correlation was found between the pKa-determined structure changes in 5'-AMP and the yield of 8,5'-cycloAMP. As the primary yield of hydroxyl radicals is relatively constant over the pH range of 3-10 (Draganic et al., 1969; Hayon, 1965), the variation in the yield of 8,5'-cycloAMP was attributed to the changing reactivity of hydroxyl radicals with the C(5') carbon in the various charged forms of 5'-AMP (Raleigh et al., 1970). Interestingly, the rotamer configuration of 5'-AMP corresponding to the (R) diastereoisomer has been found not to be the most stable conformation of 5'-AMP in free solution (Saran et al., 1972).

Irradiation of 5'-GMP, 5'-IMP and 5'-dAMP yielded chromatographic patterns suggesting the formation of other 8,5'-cyclonucleotides (Keck,

1968). However, subsequent isolation procedures were unsuccessful, and confirmation of structure was not attempted. Acid lability of the C(1') - N(9) glycosidic bond has been implicated as the reason for failure of subsequent isolation attempts (Keck, 1968). Isolation and ^{1}H-NMR characterization was possible for the 8,5'-cyclo-2'-deoxyguanosine-5'-monophosphate species resulting from irradiation of 2'-dGMP in anoxic solution (Keck, 1968). However, no diastereoisomeric classification about the C(5') carbon was reported. Radiation-chemical synthesis of 8,5'-cyclo-2'-deoxyadenosine (Mariaggi et al., 1976) was, in retrospect, reported as a stereospecific synthesis of the corresponding (R) diastereoisomer (Cadet et al., 1981).

8,5'-Cyclonucleotides and related entities offer significant advantages as models to study the biological ramifications of chemical alterations to nucleic acids in vivo. The intramolecular transfer of radiation damage initiated in the sugar phosphate moiety, to a nucleotide base as exemplified by 8,5'-cycloAMP formation, may be important in double-stranded nucleic acids where the sugar phosphate backbone is exposed to, and the bases shielded from, direct radical attack. The formation of 8,5'-cycloadenosine has been demonstrated in irradiated single-stranded DNA in vitro with yields of G(8,5'-cycloAdo) = 0.000 in N_2 and G(8,5'-cycloAdo) = 0.0015 in O_2 (Lewis and Ward, 1978). However, no attempt has been made to measure the yield of this product in vivo due to the absence of a highly sensitive assay.

The biochemical implications of 8,5'-cyclonucleotide formation on the integrity of information transfer conferred by DNA are perhaps more subtle than elimination of the base or overt strand breaks. The ability and efficiency by which the repair enzymes operate becomes a major factor

in the consequences of any type of chemical alteration to the DNA structure. The substrate specificity of various enzymes with 8,5'-cycloAMP has uncovered potentially significant data with regard to the efficiency by which enzymes repair this type of lesion. Raleigh and Blackburn (1978) were the first authors to correctly report that snake venom 5'-nucleotidase, which completely hydrolyzes 5'-AMP to 5'-adenosine, does not hydrolyze the (S) diastereoisomer of 8,5'-cycloAMP. On the other hand, the (R) diastereoisomer is hydrolyzed by 5'-nucleotidase (Hampton et al., 1972; Dudycz and Shugar, 1979). The (S) diastereoisomer has proven to be a substrate for pig muscle adenyl kinase and the resulting 8,5'-cycloAdo, a substrate for rabbit muscle pyruvate kinase (Raleigh and Blackburn, 1978). In retrospect, the (R) diastereoisomer is not a substrate for pig muscle adenyl kinase (Hampton et al., 1972). These data raise the possibility that DNA repair enzymes may selectively hydrolyze one diastereoisomer of 8,5'-cycloAMP and not the other, thus resulting in fixation of nucleotide damage.

An investigation of product formation from irradiated solutions of 5'-AMP was necessary to complement the development of an immunochemical assay to detect the 8,5'-cycloAMP moiety. Though the (S) diastereoisomer of 8,5'-cycloAMP has been shown to be a predominant product (Raleigh et al., 1976), isolation and ^{1}H-NMR characterization of the (R) diastereoisomer from irradiated solutions of 5'-AMP saturated with nitrous oxide, as presented in this thesis, has significant implications. For instance, establishing the epimeric ratio for the pH profile of 8,5'-cycloAMP formation is important for future immunological studies since, the pH within the microenvironment of the DNA helix is unknown. Even then, the

physical orientation of the base and sugar-phosphate backbone in DNA may favor formation of one diastereoisomer. With the knowledge that both epimers of 8,5'-cycloAMP result from irradiation of 5'-AMP the possibility of cross-reactivity between the two epimers in immunoassays must not be overlooked. In this context, Lewis and Ward (1978) using a phage neutralization assay to investigate the pH profile of 8,5'-cycloAMP formation, probably were unaware of the cross-reactivity with the (R) epimer that resulted in higher yields especially at pH 3.0 than obtained with an earlier HPLC analysis (Raleigh et al., 1976). Furthermore, the observation of an acid stable material exhibiting a maximum yield at pH 5.5 has significant implications in terms of the radiation chemistry of 5'-AMP and nucleic acids.

MATERIALS AND METHODS

I. DOSIMETRY

The yield of ferric ions from the oxidation of ferrous ions in an aqueous solution of ferrous sulphate salts is linearly proportional to the absorbed dose of radiation and is therefore, useful for dosimetry in radiation chemical laboratories (Fricke and Hart, 1966). The Fricke solution was prepared by dissolving 0.4 g of $FeSO_4 \cdot 7H_2O$ (AnalaR Analytical Reagent, BDH Chemicals), 0.06 g of NaCl (Reagent Grade, Chemonics Scientific) and 22.0 mL of concentrated H_2SO_4 (Baker Instra Analyzed, J.T. Baker Chemical Co.) in 1.0 L with deionized water. Two ml of Fricke solution were pipetted into each of 99 - 13 x 100 mm borosilicate glass test tubes (Fisher Laboratories Ltd.). Three test tubes were irradiated in a Gammacell-220, Co-60 gamma radiation source

(Atomic Energy of Canada Ltd.) for 0, 50, 100, 150, 200, 250, 300, 350 and 400 seconds in positions 1, 4 and 8 of the lucite test tube holder (Plate 1). The remaining seven positions were occupied by borosilicate glass test tubes containing 2.0 ml of deionized water.

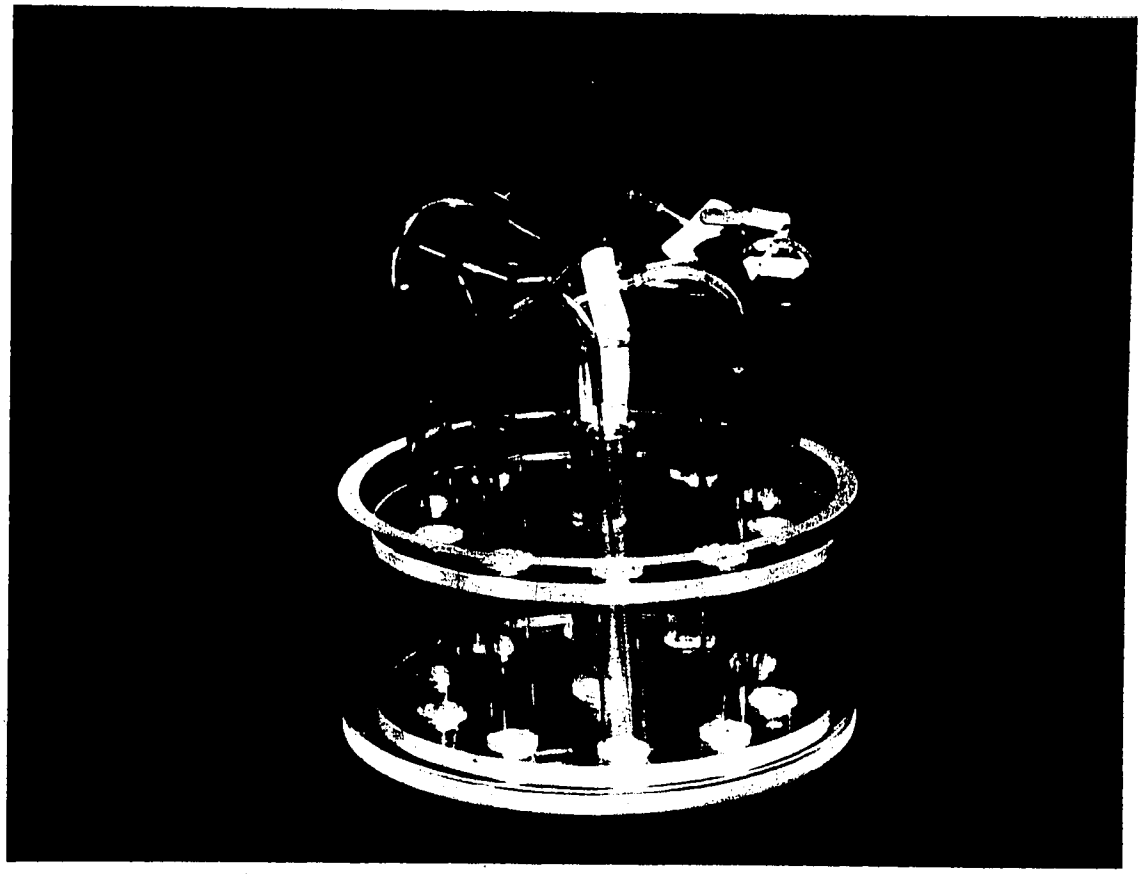

PLATE 1: Ten-test tube lucite holder used for irradiating solutions in the Gammacell-220. Solutions are bubbled by passing a stream of gas through the tubing in the center of the holder to a chamber where the gas is partitioned to capillary tubes which are immersed in the solutions. The chamber is constructed from 6-mm thick lucite and has an outside diameter of 150 mm.

Irradiations at each time interval were repeated in triplicate. The average absorbance of the irradiated solutions was measured with a Beckman DU-7 Spectrophotometer (Beckman Ind. Ltd.) operating at 305 nm and corrected by subtracting the average absorbance of unirradiated control solutions. The absorbed dose was calculated from the optical absorbance as described in Appendix A. The temperature of the solution in the spectrophotometer was 27°C.

II. HYDROLYSIS STUDY

5'-AMP (35.0 mg, 100 µmol) (Sigma Chemical Co.) was dissolved in 10.0 ml of deionized water and the pH was adjusted to 9.0 with NaOH. Two ml aliquots of this solution were added to each of 10-13 x 100 mm borosilicate test tubes.

The test tubes were placed in a lucite holder (Plate 1) and the solutions were saturated with nitrous oxide (Medigas Alberta Ltd.) by bubbling the gas through the solution 15 min before, and then throughout the time of irradiation. Nitrous oxide was scrubbed free of oxygen by passage through a 0.1 M $Na_2S_2O_4$ trap and then scrubbed free of acidic impurities in a 0.1 M Na_2CO_3 trap before entering the 5'-AMP solution. The irradiation was performed in the Gammacell - 220 to a total of 1.0 kGy at a dose rate of 79.8 Gy/min as measured by Fricke dosimetry ($G(Fe^{3+})$ = 15.6). Following irradiation, each solution was brought to 0.3 N HCl and 9 solutions were immersed in a 100°C water bath. Samples were hydrolyzed for variable lengths of time up to 55 minutes. The volume of each solution was subsequently returned to 2.0 ml by the addition of deionized water. The samples were chromatographed on a High Performance Liquid Chromatograph (HPLC) consisting

of the following components: Milton Roy Minipump with Pulse Damper, Flow rate @ 50/60 Hz, 40/400 mL/h (Bioanalytical Systems Inc.); Rheodyne Model 7120 Syringe Loading Sample Injector (Rheodyne Inc.) with a 20 µL sample injection loop; Tracor 970A Variable Wavelength Absorbance detector (Tracor Instruments) and a Spectra Physics Model 4000 Computing Integrator (Spectra Physics). Anion exchange chromatography was performed on a Partisil 10 25 PXS SAX analytical column (Whatman Inc.) monitored at 200 nm at ambient temperature and 11.0×10^{6} N/m^2 with 0.015 M K_2HPO_4 ((pH 3.1) phosphoric acid) flowing at the rate of 1.2 ml min.

III. pH STUDY

A. Calibration Curve for HPLC Analysis

A series of solutions of (S) 8,5'-cycloAMP in deionized water was prepared in the concentration range 0 - 40 µM. The optical absorbance of each solution was measured in the Beckman DU-7 Spectrophotometer at 200 nm. The concentration of (S) 8,5'-cycloAMP was calculated using the measured extinction coefficient of 14,000 M^{-1} . cm^{-1}. A 20 µL aliquot of (S) 8,5'-cycloAMP was injected into the HPLC (see Section II) and the observed peak area of (S) 8,5'-cycloAMP was plotted as a function of the concentration. The calibration curve was employed to determine the concentration of 8,5'-cycloAMP from the peak area as measured by HPLC. Due to the limited quantities of pure (R) epimer available, the calibration curve derived for the (S) diastereoisomer was used to quantitate the yield of both epimers in the irradiated solutions.

B. pH Profile for the Formation of 8,5'-cycloAMP

A stock solution of 5'-AMP was prepared by dissolving 71.5 mg (200 µmol) of 5'-AMP into 20.0 mL of deionized water. Solutions with different pH values in the range 2 - 11 were prepared by adding 1.0 mL of the stock solution of 5'-AMP to 8.0 mL of deionized water in a 10.0 mL beaker. Adjustment of the pH of the solution was made by adding dilute sodium hydroxide or perchloric acid. Hydrochloric acid may be substituted for perchloric acid with identical results (Fuciarelli and Raleigh, unpublished observations). The pH of the solution was monitored by means of a Corning 130 pH meter (Corning Glass Works of Canada Ltd.). The pH meter was calibrated with: 1) pH Reference Buffer Solution pH 4.01 $\pm$ 0.01 @ 25^{o}C (Canlab) in the range 2 - 5 and 2) Standard Buffer Solution pH 7.00 $\pm$ 0.01 @ 25^{o}C (Sargent Welch) in the range 6 - 11. The solution was transferred to a 10.0 mL volumetric flask and brought to 10.0 mL with deionized water. At this point, the final pH of the solution was measured. Two mL of each solution were placed in 30 - 13 x 100 mm borosilicate glass test tubes. Triplicate samples were irradiated for each pH. Gas saturation with nitrous oxide, irradiation and HPLC analysis were the same as described in Section II. Samples were hydrolyzed for 60 minutes in 0.3 N HCl and returned to 2.0 mL with deionized water prior to HPLC analysis. The concentrations of the (R) and (S) diastereoisomers of 8,5'-cycloAMP were calculated from the calibration curve obtained from Section III-A. The integral peak area was plotted as a function of known concentrations of pure (S) 8,5'-cycloAMP as measured by a UV spectrophotometer in the relevant concentration range. The molar

extinction coefficient was measured for (S) 8,5'-cycloAMP and the assumption was made that the (R) diastereoisomer would have a similar extinction coefficient.

IV. RADIATION CHEMICAL SYNTHESIS OF 8,5'-cycloAMP

In a representative radiation chemical synthesis of the (S) and (R) diastereoisomers of 8,5'-cycloadenosine-5'-monophosphate, 500 ml of deionized water containing 18.0 gm of 5'-AMP were irradiated at pH 9.0 to a total dose of 100 kGy in a 500 ml Erlenmeyer flask.

The irradiated solution was chromatographed in 105 ml portions on a 200 - 400 mesh AG1-X8 (formate) column (4.5 x 28 cm) (BioRad Laboratories Ltd.) with 0.4 N or 0.6 N formic acid as eluants. Fifty ml fractions were collected with a BioRad Model 1320 Fraction Collector (BioRad Laboratories Ltd.).

The fractions were monitored using a High Performance Liquid Chromatograph (HPLC) consisting of the components indicated in Section II with the substitution of a Fisher Recordall Series 5000 Chart Recorder (Fisher Lab. Ltd.) for the computing integrator. Anion exchange chromatography was performed as previously described in Section II. Fractions were pooled according to differential retention times and brought to dryness with a Büchi Rotovaporator RE 120 (Büchi Laboratoriums - Technik AG) operating in vacuo at 40°C. Fractions containing mixtures of products were subsequently rechromatographed. Products of the radiation chemical synthesis were chromatographed on a 200 - 400 mesh Dowex W-X4 (hydrogen) column (2.5 x 15 cm) (Dow Chemical Co.) to ensure removal of cationic contaminants with deionized water being used as the eluant. The products were brought to dryness in vacuo and stored over P_2O_5 in a vacuum dessicator.

A. Chemical Analysis

Infrared spectra were recorded on a Unicam SP1050 Infrared Spectrophotometer (Pye-Unicam Ltd.) in KBr pellets. Ultraviolet spectra were recorded on a Pye Unicam SP8-100 Ultraviolet Spectrophotometer (Pye Unicam Ltd.) or a Beckman DU-7 UV/Vis Spectrophotometer. Melting points were recorded on a Fisher Digital Melting Point Analyzer Model 355 (Fisher Scientific Ltd.).

B. High Resolution Mass Spectrometry (HRMS)

For HRMS, 8,5'-cycloAMP was dephosphorylated by alkaline phosphatase to yield 8,5'-cycloadenosine. In a representative experiment, 42.0 mg of (S) 8,5'-cycloAMP was dissolved in 5.0 mL of Sørenson's glycine buffer II, pH 10.4, containing 10.0 mg of alkaline phosphatase (Sigma Chem. Co., 1.1 U/mg). The solution was incubated at 37°C and the reaction was judged complete by monitoring the disappearance of 8,5'-cycloAMP by HPLC. (S) 8,5'-Cycloadenosine crystallized out of solution, was pelleted by centrifugation, washed twice with deionized water and then dried with nitrogen gas after the supernatant was drawn off.

High resolution mass spectrometry was performed on a Kratos MS 50 mass spectrometer.

C. Nuclear Magnetic Resonance Spectroscopy

The ^{1}H-NMR spectra were recorded at 200 MHz on a Bruker WH-200 instrument or at 400 MHz on a Bruker WH-400 instrument. The proton chemical shifts in $^{2}H_2O$ were measured vs. internal calibration of the resonance resulting from H_2O and in $(C^{2}H_3)_2SO$ vs. internal Me_4Si to an accuracy of $\pm$0.01 ppm. The coupling constants, $J(^{1}H, ^{1}H)$ were determined

to an accuracy of 0.01 Hz. Samples were analyzed at a concentration of 10^{-2} M and a temperature of 300 ^{o}K.

RESULTS

I. DOSIMETRY

The total dose, plotted as a function of the time of irradiation, is illustrated in Figure 5. Each data point represents the average absorbed dose for three measurements. The standard error about the mean of each data point falls within the boundaries of the symbols. A linear relationship exists between the total absorbed dose and the time of irradiation. This relationship may be described by the equation: $y = 0.133x + 1.000$ with a correlation coefficient of 0.9999 as determined by regression analysis. A dose of approximately 10.0 Gy is delivered to the solutions as the irradiation chamber raises and lowers the samples into the source within the Gammacell. As determined from Figure 5, the dose rate determined for the lucite test tube holder under conditions modelling the subsequent radiation chemistry experiments (with the exception that nitrous oxide gas was not bubbled through the Fricke solutions) was 79.[illegible] min.

II. HYDROLYSIS STUDY

Irradiation of an aqueous solution of 5'-AMP with nitrous oxide saturation at pH 9.0 to 1.0 kGy, results in the formation of a number of products which may be resolved by high pressure liquid chromatography as demonstrated in Figure 6. Characterization of the material responsible for selected peaks was accomplished using ^{1}H-NMR spectroscopy - the results of which are described in Section IV-B. Acid

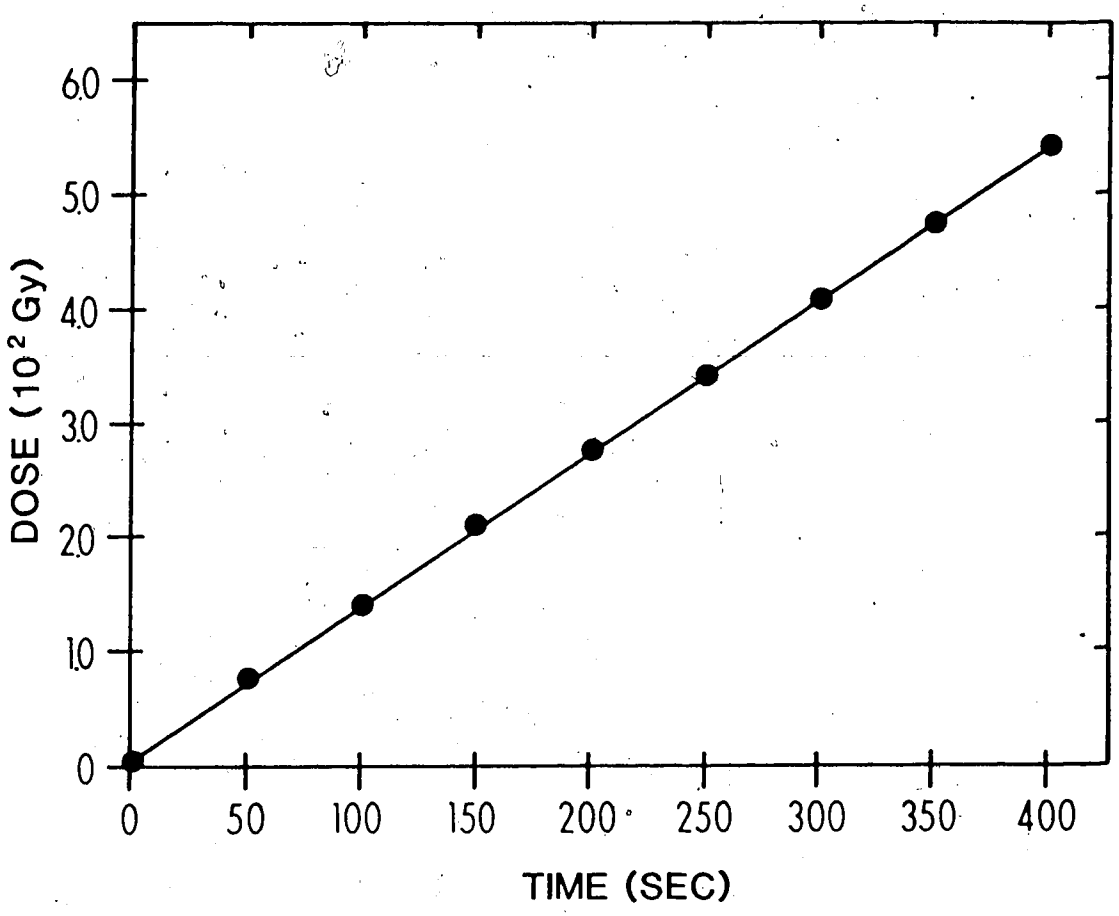

FIGURE 5: Fricke dosimetry for the lucite holder in the Gammacell-220. Each data point represents the mean of three samples. The range for the standard error lies within the boundary of each point. The dose rate was measured as 79.8 Gy/min.

hydrolysis (0.3 N HCl at 100°C) of unchanged 5'-AMP to adenine was necessary to resolve the (S) epimer of 8,5'-cycloAMP from 5'-AMP. Figure 6 illustrates the effect of hydrolysis on the products in the irradiated solution over a period of 55 minutes. Both the (S) and (R) epimers of 8,5'-cycloAMP are acid stable, whereas material eluting with a slower retention time under Peak X consists of an acid labile component which disappears within five minutes, as well as an acid stable component. Another acid labile component has a retention time slightly shorter than 5'-AMP (Figure 6). After five minutes of acid

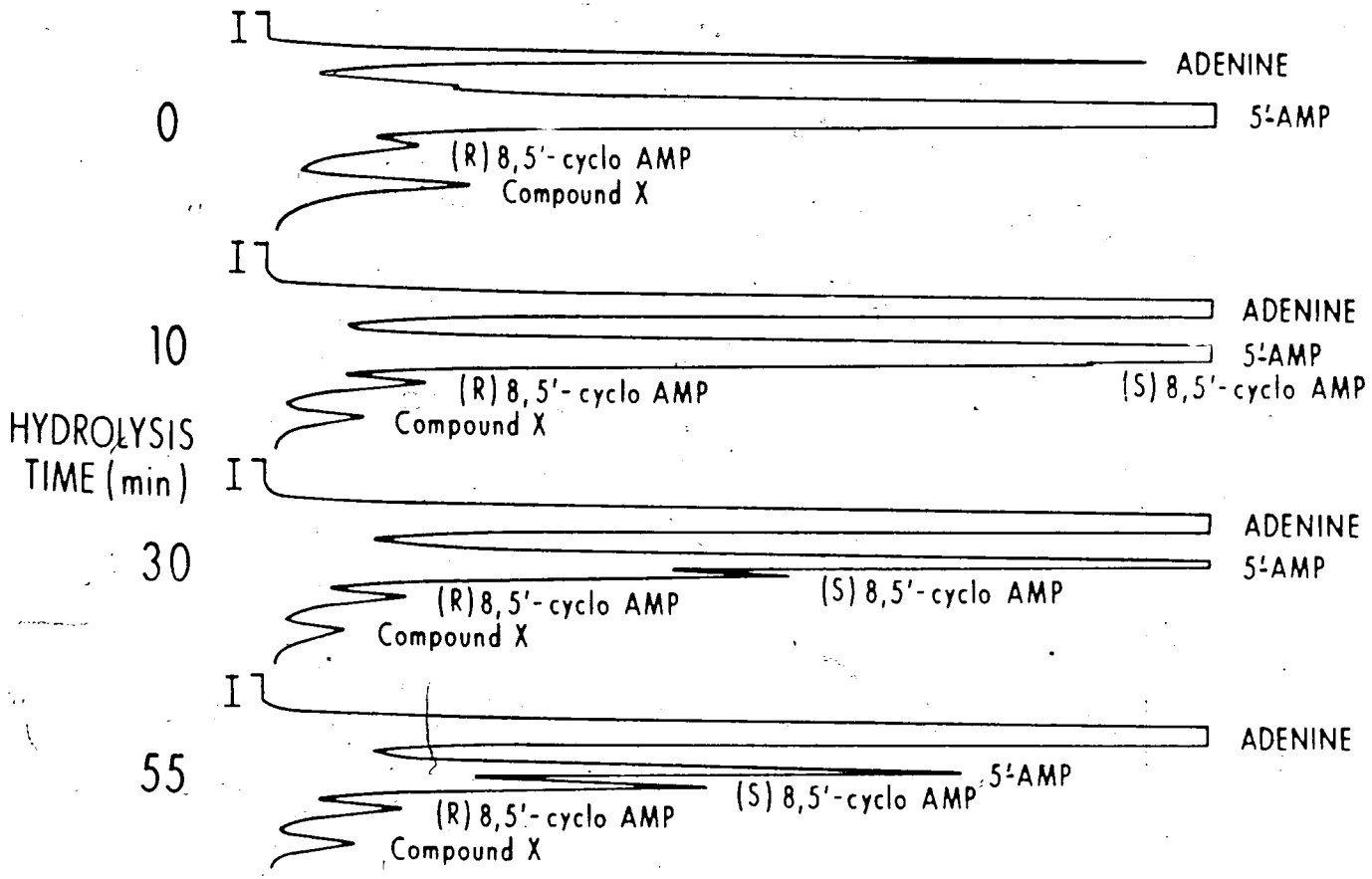

FIGURE 6: Hydrolysis study of irradiated 5'-AMP solutions. Ten mM solutions of 5'-AMP were saturated with nitrous oxide then irradiated to 1.0 kGy. Samples of 2.0 mL were brought to 0.3 N HCl and hydrolyzed at 100°C for varying lengths of time then chromatographed by HPLC.

hydrolysis, the acid labile components are not detectable. Both diastereoisomers of 8,5'-cycloAMP and the acid stable component eluting at Peak X are stable to hydrolysis under these experimental conditions for at least 55 min.

III. pH STUDY

A. Integral Peak Area

With the assurance that 8,5'-cycloAMP was stable to acid hydrolysis, the pH profile of 8,5'-cycloAMP formation was investigated to determine the pH at which the yield of this species was maximized during the radiation chemical synthesis. Figure 7 illustrates the mean integral peak area of 3 measurements (± standard error) plotted as a function of the pH of the solution. The acid stable, uncharacterized material eluting at Peak X, exhibits a maximal yield at pH 5.5. The yield of the 8,5'-cycloAMP diastereoisomers shall be discussed in terms of the product yields in Section III-C.

B. Calibration Curve for HPLC Analysis

The peak area obtained from HPLC analysis is plotted as a function of the concentration of (S) 8,5'-cycloAMP in Figure 8. Regression analysis reveals a linear relationship best described by the relationship: $y = 347.70x + 62.92$ with a correlation coefficient of 0.9988. The yield of 8,5'-cycloAMP may then be expressed in terms of the G-value which is calculated by dividing the micromolar concentration of the solution by the dose in daGy. This analysis has been applied to the yield of 8,5'-cycloAMP in Section III-C.

C. Product Yield

The pH profile for the formation of 8,5'-cycloAMP is shown in Figure 9 which illustrates the G-value of 8,5'-cycloAMP plotted as a

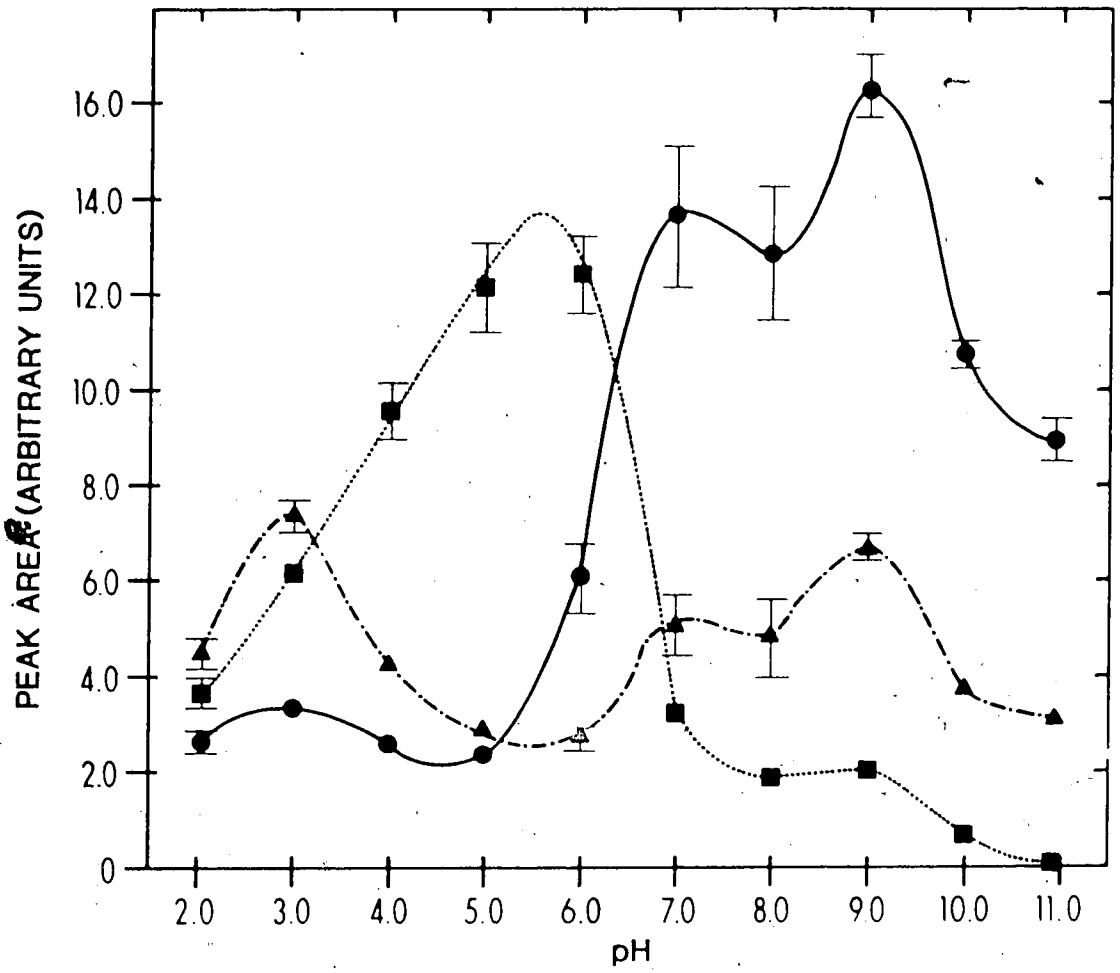

FIGURE 7: pH Profile of product formation in irradiated solutions of 5'-AMP. Millimolar solutions of 5'-AMP, the pH of which was varied with perchloric acid and/or sodium hydroxide, were saturated with nitrous oxide, irradiated to 1.0 kGy, hydrolyzed for 60 min (0.3 N HCl at 100°C) and chromatographed by HPLC. Each data point represents the average integral peak area of three samples (± standard error). (S) 8,5'-cycloAMP (●), (R) 8,5'-cycloAMP (▲), Compound X (■).

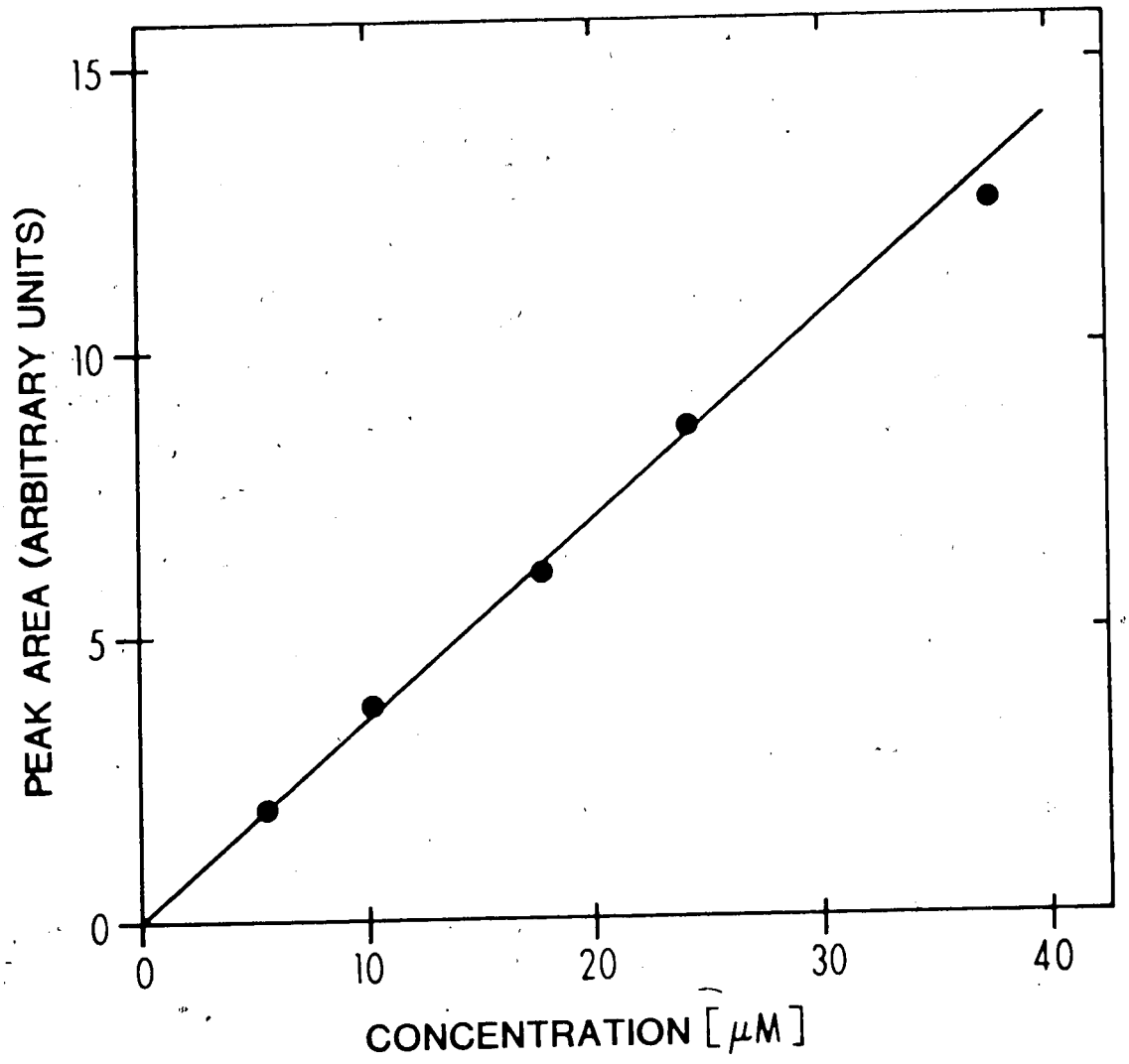

FIGURE 8: Calibration curve for HPLC analysis. The concentration of (S) 8,5'-cycloAMP was measured in a spectrophotometer prior to injection into the HPLC. The concentration of samples containing either diastereoisomer of 8,5'-cycloAMP may be determined from the integral peak area of the HPLC chromatogram.

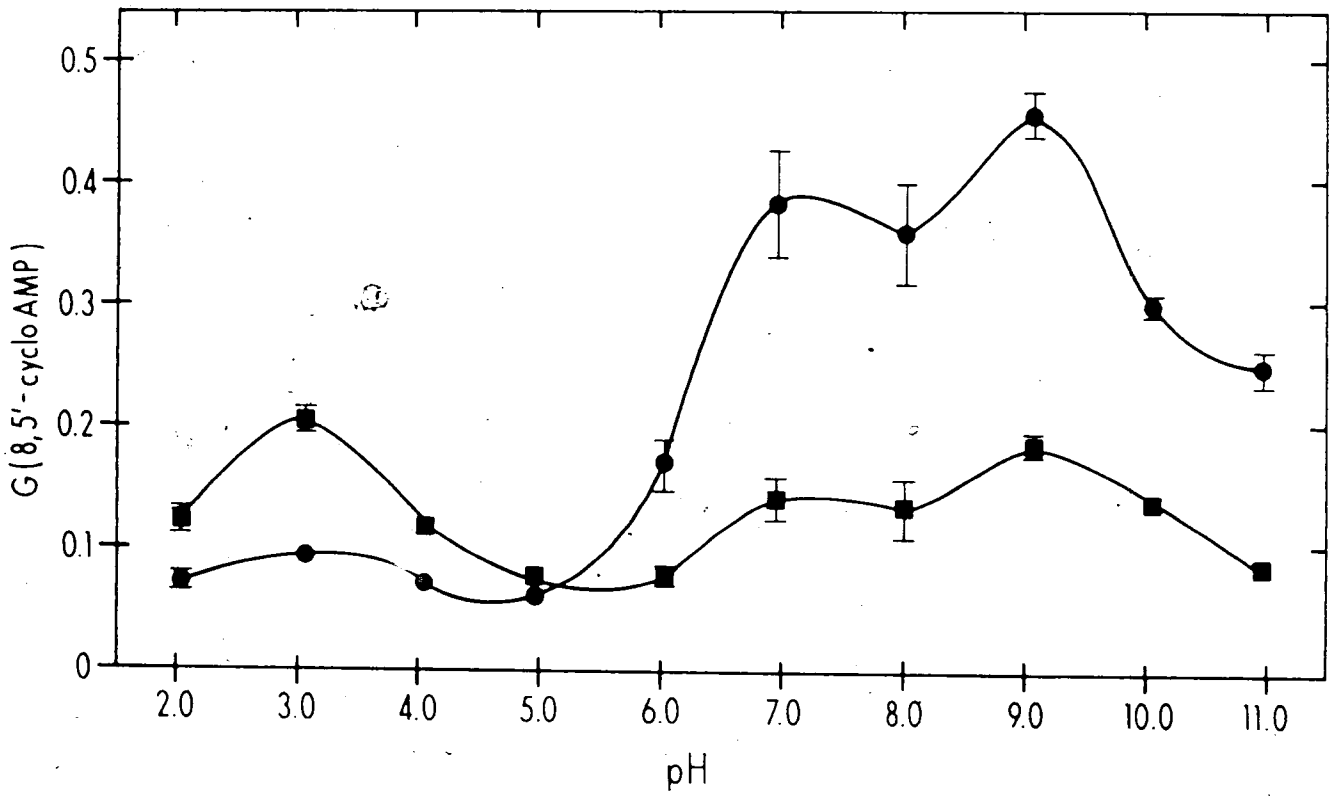

FIGURE 9: pH Profile of product formation in irradiated solutions of 5'-AMP. Millimolar solutions of 5'-AMP, whose pH was adjusted with perchloric acid and/or sodium hydroxide, were saturated with nitrous oxide, irradiated to 1.0 kGy, hydrolyzed for 60 min (0.3 N-HCl at 100°C) and chromatographed by HPLC. Each data point represents the average G-value of three samples (± standard error). (S) 8,5'-cycloAMP (●). (R) 8,5'-cycloAMP (■).

function of the pH after 60 minutes of acid hydrolysis. Each data point represents the mean integral area of 3 samples ± standard error about the mean. Maxima in the formation of 8,5'-cycloAMP were found at pH 3.0, 7.2 and 9.0. At pH 7.2 and 9.0 the (S) epimer is produced at approximately 2.5 x the yield of the (R) epimer. In contrast, at pH 3.0, the (R) epimer is produced at twice the yield of the (S) epimer. The yield of both diastereoisomers of 8,5'-cycloAMP are formed with low yields (G-value approximately 0.060 - 0.070) in the pH range of 4.50 - 5.70. The minimum in the yield at pH 8.0 has been demonstrated in a number of experiments, including an earlier publication by Raleigh et al., 1976.

IV. RADIATION CHEMICAL SYNTHESIS OF 8,5'-cycloAMP

An irradiated solution of 5'-AMP was chromatographed in fractions. Table 3 represents the elution profile of two such chromatographs. In irradiation A, the irradiated solution was chromatographed in two fractions with 0.4 M HCOOH as an eluant. The components were generally resolved from each other. If the eluant is raised to 0.6 M HCOOH the components are less likely to be well resolved, though the total elution time is decreased. Products co-eluting in Peak X were not followed in these representative syntheses but are easily removed by continuing the flow of eluant.

A. Analytical Data

(S) 8,5'-Cycloadenosine-5'-monophosphate. The sample selected for analytical work was chromatographically pure (HPLC), m.p. 145-155°C (dec); UV $\lambda_{max}^{pH\ 7.0}$ 266 nm (ε = 14,000 x 10^3 M^{-1} . cm^{-1}); NMR: refer to Section IV-B; IR KBr, (cm^{-1}) 1710, 1620, 1460; m/e (nucleoside) 265 (see Figure 10).

TABLE 3: PREPARATIVE CHROMATOGRAPHY OF IRRADIATED 5'-AMP SOLUTIONS

Irradiation A

Column Flow Rate: 7.0 mL/min

Solvent: 0.4 M HCOOH

	Volume (mL)	
Product	A	B
Adenine	0-1500	0-1900
5'-AMP	1500-2400	1900-2900
(S) 8,5'-cycloAMP	2400-3200	2900-3400
(R) 8,5'-cycloAMP	3200-3900	3400-4300

Irradiation B

Column Flow Rate: 6.8 mL/min

Solvent: 0.6 M HCOOH

	Volume (mL)			
Product	A	B	C	Average
Adenine	0-700	0-600	0-600	(0-600)
5'-AMP	700-1400	600-1400	600-1350	(600-1400)
(S) 8,5'-cycloAMP	1400-1950	1400-1900	1350-2050	(1400-2000)
(R) 8,5'-cycloAMP	1950-2500	1900-2500	2050-2400	(2000-2500)

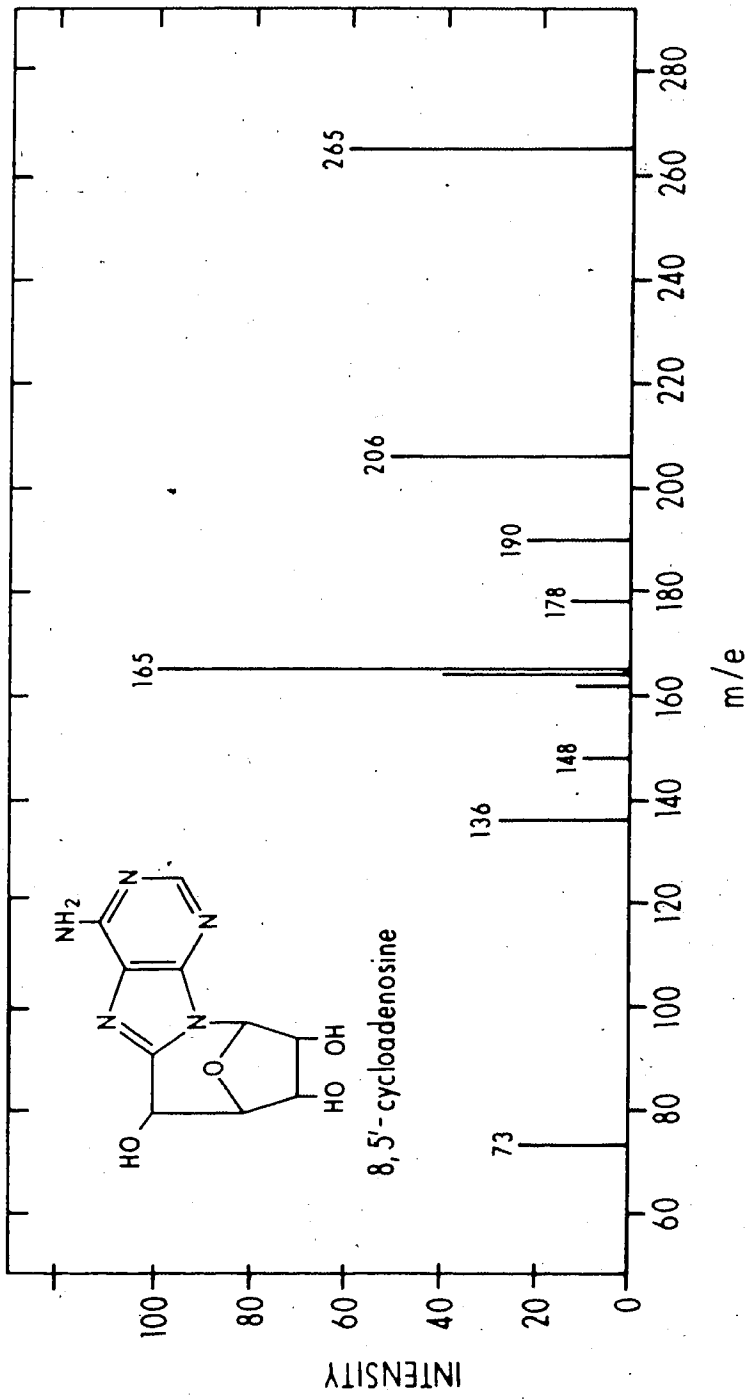

FIGURE 10: Mass spectrum of (S) 8,5'-cycloadenosine. Only ions exhibiting greater than 10% relative abundance are illustrated.

(R) 8,5'-Cycloadenosine-5'-monophosphate. The sample selected for analytical work was greater than 95% pure (HPLC), m.p. 240°C (dec); UV $\lambda_{max}^{pH\ 7.0}$ 266 nm; NMR: refer to section IV-B.

B. Nuclear Magnetic Resonance Spectroscopy

The results of analyses of the ^{1}H-NMR spectra for two products isolated from irradiation of 5'-AMP at pH 9.0 are tabulated in Tables 4 and 5 and illustrated in Figures 11 and 12. Assignment of the low field singlets to H(2) and H(1') is by analogy with 5'-AMP. Absence of H(8) is indicative of the presence of a heterocyclic ring resulting from formation of a C(5') to C(8) bond. Identification of two diastereoisomers, namely the (R) and (S) epimeric cyclonucleotides was facilitated by the ^{1}H-NMR signal for the C(5') proton.

The nomenclature adopted in this presentation refers to that used to describe rotamers about the C(4') - C(5') bond in NMR studies of nucleotides which possess free rotation about the C(4') - C(5') bond. Theoretically, the distinction between the (R) and (S) diastereoisomers can be made a priori. The (R) and (S) epimeric cyclonucleotides exhibit exocyclic group conformations which differ from the classical gauche-trans and trans-gauche configurations by about 20° (angle about the C(4') - C(5') bond) which is a result of the intramolecular C(5') to C(8) bond and permits unequivocal identification of the two diastereoisomers. Following general Karplus coupling constant rules, the gauche-trans conformation should show a coupling constant close to zero, as the dihedral angle is close to 90°. By contrast, the trans-gauche configuration should show a substantial coupling constant between H(4') and H(5'). Experimental results are in agreement with these predictions.

TABLE 4: PROTON CHEMICAL SHIFTS

Nucleotide	Solvent	H(2) ppm	H(8)	H(1')	H(2')	H(3')	H(4')	H(5')	H(5'')	NH_2
5'AMP[a]	^2H_2O	8.01	8.38	6.06	4.76	4.50	4.40	4.10	4.10	-
(S) 8,5'-cycloAMP	^2H_2O	8.32	-	6.26	4.37	4.71	4.01	5.66	-	-
(S) 8,5'-cycloAMP	$(C^2H_3)_2SO$	8.16	-	6.02	4.04	4.48	4.83	5.60	-	7.42
(R) 8,5'-cycloAMP	^2H_2O	8.42	-	6.40	4.31	4.38	4.02	5.38	-	-
(R) 8,5'-cycloAMP	$(C^2H_3)_2SO$	8.22	-	6.11	3.07	4.21	4.01	5.30	-	7.60

[a] from Stolarski et al. (1080)

TABLE 5: VALUES OF PROTON VICINAL COUPLING CONSTANTS

Nucleotide	Solvent	J(1',2')	J(2',3')	J(3',4')	J(4',5')	J(4',5")	J(5',5")	$J(^{31}P,5')$	$J(^{31}P,5")$
		Hz							
5'AMP[a]	^2H_2O	5.4	5.0	3.9	3.9	2.6	11.8	4.6	4.6
(S) 8,5'-cycloAMP	^2H_2O	0.0	-	0.0	5.0	-	-	6.4	-
(S) 8,5'-cycloAMP	$(C^2H_3)_2SO$	0.0	0.0	0.0	5.7	-	-	8.4	-
(R) 8,5'-cycloAMP	^2H_2O	0.0	5.8	0.0	-	-	-	-	0.0

[a] from Sarma et al. (1974)

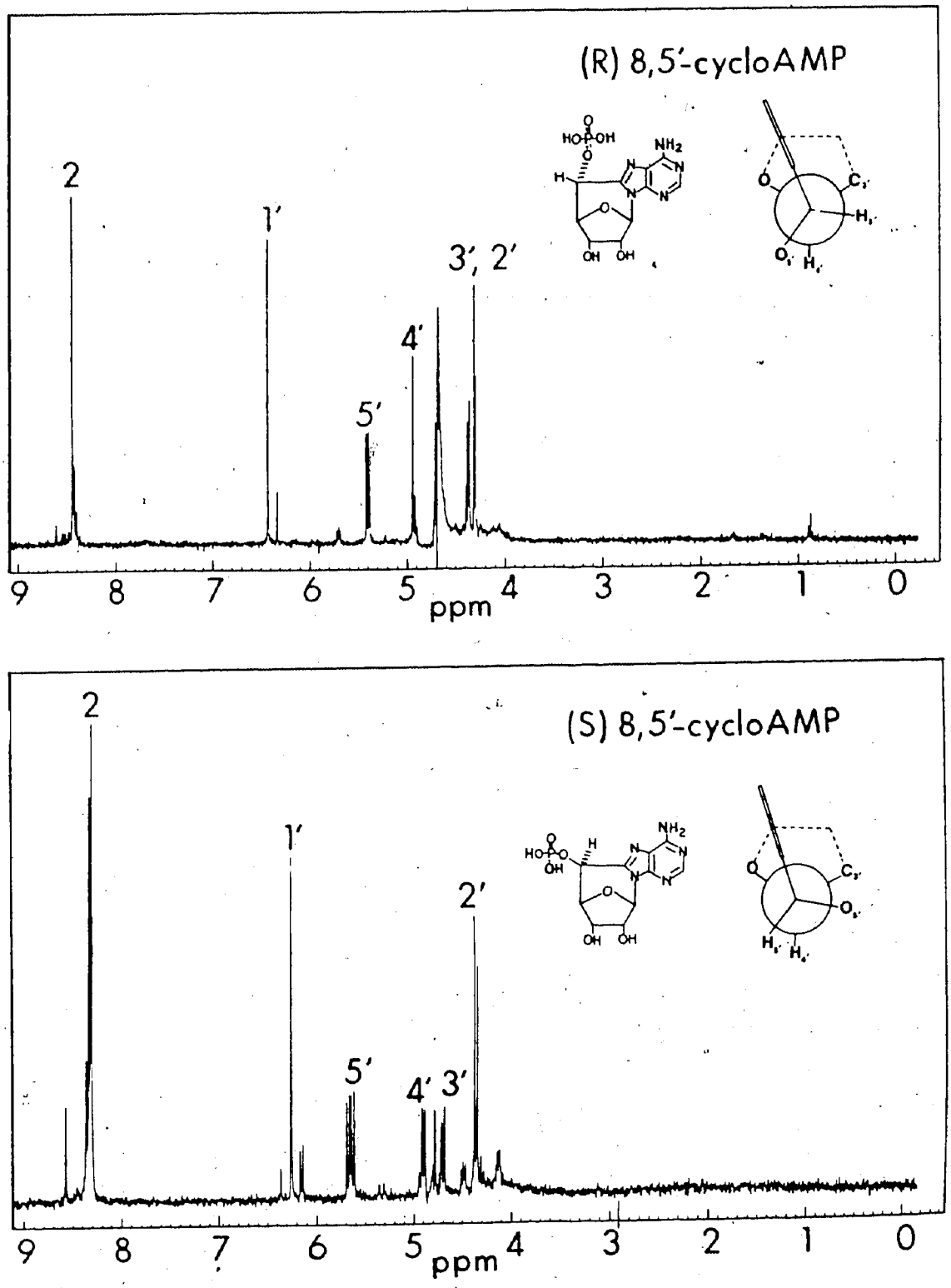

FIGURE 11: 200 MHz NMR Spectra of 8,5'-cycloAMP in $^{2}H_2O$. The sample concentration was 10^{-2} M and the temperature was 300°K.

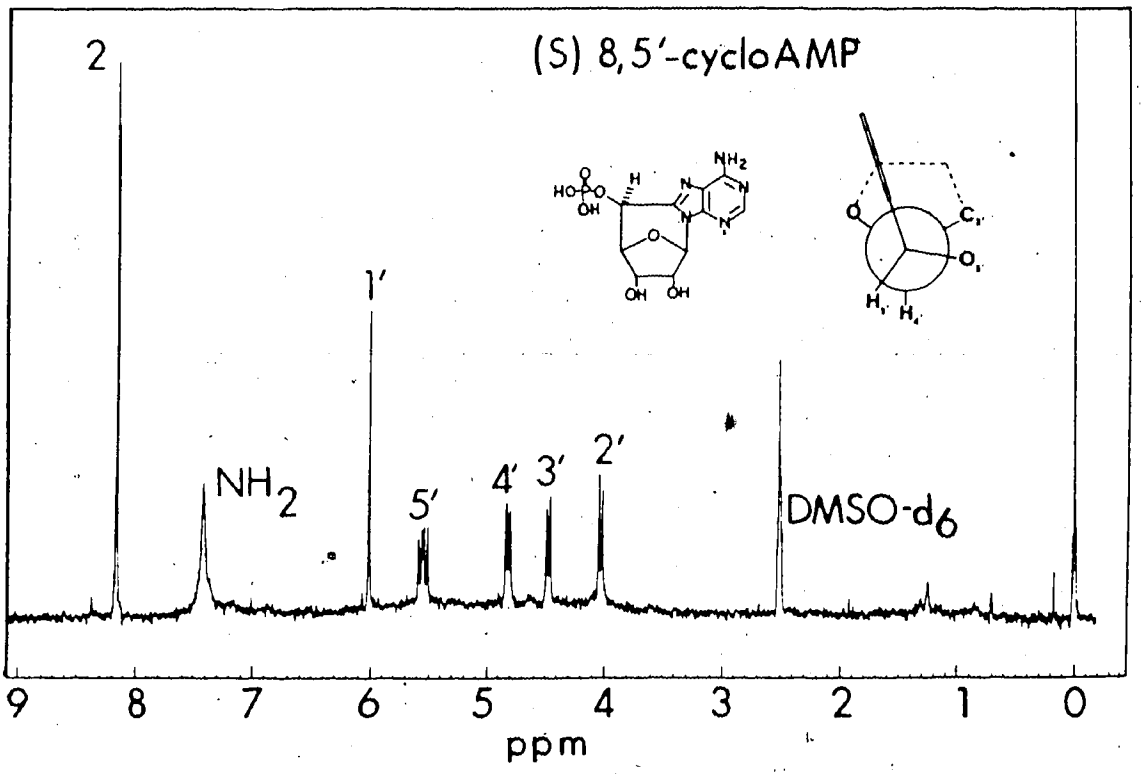

FIGURE 12: 200 MHz NMR Spectrum of (S) 8,5'-cycloAMP in $(C^2H_3)_2SO$. The concentration of the sample was 10^{-2} M and the temperature was 300°K.

A comparison of the (R) and (S) diastereoisomers may be made at the H(5') resonance peak of a 10^{-2} M solution of each epimer in ^2H_2O at pH 7.0 (Figure 11, Tables 4 and 5). The (R) epimer is identified by a doublet splitting of 9.0 Hz at 5.38 ppm due to coupling to the phosphate phosphorus group and a relatively small secondary splitting of 0.3 Hz by H(4') for which the resonance appears as the singlet at 4.92 ppm. By contrast, (S) 8,5'-cycloAMP exhibits a doublet of doublets at 5.66 ppm with a primary splitting of 9.4 Hz due to phosphate phosphorus coupling to H(5') and a secondary splitting of 5.9 Hz due to coupling to H(4'), for which the resonance appears as the doublet (5.8 Hz) at 4.91 ppm. Assignment of the H(2') or H(3') protons to specific resonance peaks in the high field is not possible in ^2H_2O.

The highest field resonances with a splitting of 5.8 Hz are attributed to H(2') and H(3') though assignment of individual peaks is controversial. Despite arguments presented by Stolarski et al. (1980), their assignment of the high field resonance peaks is not supported by either theoretical or experimental arguments. As for the theoretical argument, Haromy et al. (1980) demonstrated that in the (S) diastereoisomer of 8,5'-cycloadenosine, the hydroxyl group is oriented toward the ribose ring. It follows, therefore, that in the case of the nucleotide, the phosphate group has the potential to deshield the H(3') proton due to the proximity of the phosphorus group to the H(3') proton. In this chemical environment, the resonance peak contributed by the H(3') may be expected to have a slight downfield shift which has been observed in the $(C^2H_3)_2SO$ spectrum (Figure 12). Similar arguments would contend that the phosphate group would not significantly shift the H(3') resonance peak in the (R) diastereoisomer since the phosphate group is not lying over the ribose ring, based on the observation that in the nucleoside, the hydroxyl group is oriented away from the ribose ring (Birnbaum et al., 1981). On an experimental basis, assignment of the H(2') and H(3') protons to a specific resonance peak may be achieved by a spin decoupling experiment. Since one of the high field resonance peaks was slightly broader than the other, spin decoupling experiments were performed to determine if this broadening was the result of coupling between specific protons. As a result of irradiation of the H(4') proton, there is a 27% reduction in the width of the resonance peak at 4.48 ppm as compared to a 6.2% reduction in the peak at 4.04 (Table 6). Therefore, the H(3') proton may be assigned to the resonance at 4.48 ppm and the H(2') proton to the resonance at 4.04 ppm.

TABLE 6: SPIN DECOUPLING EXPERIMENT TO ESTABLISH ASSIGNMENT OF THE H(2') AND H(3') PROTONS TO NMR RESONANCE PEAKS FOR (S) 8,5'-cycloAMP

Solvent: $(C^2H_3)_2SO$

	Resonance Peak Width (Hz)	
	ppm	
	4.04	4.48
Blank	2.00	2.05
Irradiated at 4.83 ppm (H(4'))	1.88	2.14
Percent Change	6.2	27

DISCUSSION

Studies on the radiation chemistry of anaerobic solutions of 5'-AMP initiated by the pioneering work of Keck (1968) and Raleigh et al. (1976) have revealed that the predominant product formed is the (S) diastereoisomer of 8,5'-cycloAMP (Haromy et al., 1980). The work presented as part of this thesis is in agreement with these findings, but also illustrates that a number of other products are formed. One product, formed at a lower yield than (S) 8,5'-cycloAMP, has been unequivocally identified as the (R) diastereoisomer of 8,5'-cycloAMP.

The acid stability of (S) 8,5'-cycloAMP was established during earlier work (Keck, 1968; Raleigh et al., 1976). However, the relative stability to acid hydrolysis for (R) 8,5'-cycloAMP and other products was investigated by acid hydrolysis studies. Acid hydrolysis of 5'-AMP results in fragmentation of the nucleotide due to cleavage of the C(1') - N(9) glycosidic bond and liberation of the base adenine. On the other hand, both diastereoisomers of 8,5'-cycloAMP, and an unidentified material (Compound X) are stable to the conditions of hydrolysis reported herein. There is evidence for at least two acid labile components, one compound eluting at the same position as Compound X with analytical HPLC, and another eluting slightly faster than 5'-AMP. With preparative chromatography, Compound X and this labile material may be separated (Fuciarelli and Raleigh, unpublished observations). Acid hydrolysis does not, by itself, result in the formation of either diastereoisomer of 8,5'-cycloAMP or Compound X since no increase in the yield of these products occurs during the course of hydrolysis.

Raleigh et al. (1976) observed a close correlation between the pKa-determined structure changes in 5'-AMP (Fig. 13) and the yield of

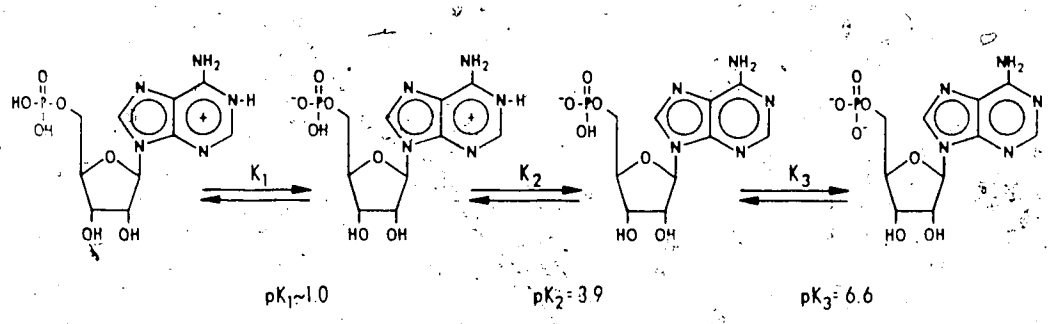

FIGURE 13: pKa-determined structure changes in 5'-AMP.

(S) 8,5'-cycloAMP. A similar relationship has been observed with both the (R) and (S) diastereoisomers of 8,5'-cycloAMP in the present work, although the yield of the (S) diastereoisomer at pH 3.0 is lower (G 0.10) than the yield reported earlier (G 0.31) (Raleigh et al., 1970). Moreover, there is a more pronounced minimum in the yield of the (S) epimer at pH 8.0 than reported earlier (Raleigh et al., 1970).

The pH profile for the formation of both diastereoisomers of 8,5'-cycloAMP (Figure 9) reveals that there is a decreasing reactivity of the hydroxyl radical with 5'-AMP below pH 2.0 and above pH 11.0. The decreasing reactivity below pH 2.0 is reproducible when either perchloric or hydrochloric acid is used to adjust the pH of the solution - an observation which supports the earlier work of Raleigh et al., (1970), and is correlated with complete protonation of the 5'-AMP moiety (Figure 13, pK_1 approximately 1.0). The reduction in the yield of 8,5'-cycloAMP at high pH has been attributed to the decreased reactivity of O^- ($HO \rightleftharpoons O^- + H^+$, pKa = 11.9) with C(5') in the doubly charged 5'-AMP moiety (Raleigh et al., 1970).

Since the yield of hydroxyl radicals is relatively constant over the pH range 2 - 10 (Hayon 1965; Draganic et al., 1969) the total yield of hydroxyl radical-induced damage, including possible back reactions (Conlay, 1963), should be constant. In this context, the variation in yield of the products resulting from irradiation of 5'-AMP with the pH is most interesting. The minimum in formation of both diastereoisomers of 5'-AMP at pH 4.5 - 5.5 corresponds with a maximum in the formation of Compound X (Figure 7). This observation may reflect the changing reactivity of the hydroxyl radical at the C(5') position

of the sugar and the C(8) position of the base, which would be consistent with earlier predictions (Raleigh et al., 1970).

In light of the demonstration in the present work of the radiation-induced formation of both diastereoisomers of 8,5'-cycloAMP, previous claims of stereospecific radiation chemical synthesis of cyclonucleotides (Raleigh et al., 1970; Raleigh and Blackburn, 1978; Cadet et al., 1981 and possibly Keck, 1968) must be amended to stereoselective radiation chemical synthesis. Such syntheses are stereoselective, in that they exhibit a higher yield of one species over other possible species, as a direct consequence of the pH of the solution at the time of irradiation.

In summary, the radiation chemistry of 5'-AMP has been investigated in an attempt to characterize the products which might result from adenine-based species in polynucleotides in conjunction with the development of an immunochemical probe for the 8,5'-cycloAMP moiety.

CHAPTER 3:

DEVELOPMENT OF AN IMMUNOCHEMICAL ASSAY WITH SPECIFICITY TO 8,5'-cycloAMP RADIATION-INDUCED CHEMICAL MODIFICATIONS

INTRODUCTION

The impetus for immunochemical research emanated from the pioneering work of Landsteiner (1945) who initially demonstrated that a multitude of organic compounds of low molecular weight could be rendered antigenic after conjugation to suitable protein "carriers". Immunoassays have been developed with specificity for a variety of nucleic acid constituents including purine and pyrimidine bases, nucleosides, nucleotides, small oligonucleotides and nucleic acids (see reviews by Beiser and Erlanger, 1960; Plescia and Braun, 1967; Levine and Stollar, 1968; Butler and Beiser, 1973; Lacour et al., 1973; Stollar, 1975, 1980; Munns and Liszewski, 1980).

The quality of an immunoassay is inherently a function of the quality of the antiserum produced as defined by the following parameters: 1) antibody concentration, which reflects the concentration of antibodies that recognize a specific antigenic determinant; 2) antibody avidity (or affinity), which is an expression of the energy of the reaction between the determinants and the combining sites of the antibodies and defines the ultimate sensitivity of an immunoassay and 3) antibody specificity, which is a measure of the degree of exclusivity by which the antibodies recognize an antigenic determinant. Since polyclonal antisera contain a heterogeneous population of antibodies, the avidity and specificity reflect average measures of this heterogeneity.

In light of the vast quantity of literature addressing many of the techniques available for the development of an immunochemical assay, a comprehensive survey shall not be attempted. However, the development of an assay for 3',5'-cycloAMP involved strategic decisions at three phases. The rationale underlying these decisions shall be briefly discussed. The three strategic phases are:

1) Synthesis of an immunogen by conjugation of the hapten via a functional group to a protein carrier.
2) Induction of anti(protein-hapten) antibodies by the selection of a suitable immunogen dose and time course for inoculation.
3) Characterization of the antisera to evaluate the specificity and sensitivity of an immunoassay and the optimization of various parameters involved in the immunoassay.

1. Synthesis of the Immunogen

Antibodies with specific determinants to nucleic acid constituents may be elicited by synthetic protein-hapten conjugates. The chemistry involved in the synthesis of a covalently linked protein-hapten conjugate has been extensively reviewed (Parker, 1971; Erlanger, 1973; Becker, 1978; Erlanger, 1980). Two techniques used to prepare immunogens containing nucleotide analogues are the periodate oxidation technique (Erlanger and Beiser, 1964) and the imidazolide method (Johnston et al., 1983).

The universal acceptance of the periodate oxidation technique, as originally described by Erlanger and Beiser (1964) is based upon: 1) the simplicity of the reactions involved; 2) the variety of nucleoside and nucleotide constituents amenable to this form of conjugation; 3) the degree to which the various haptens can be conjugated per molecule of

carrier protein and 4) the lack of significant degradation or modification of the base during conjugation (Munns, 1977). The reaction, as outlined in Figure 14, utilizes the vicinal hydroxyl groups contained in the ribose moiety. Oxidation of these hydroxyl groups with periodate (IO^-_4) yields a nucleoside-dialdehyde structure that readily undergoes condensation with primary amino groups (i.e. the ε-NH_2 group of lysine present in the carrier protein). The resulting Schiff-type bases are reduced with borohydride into stable compounds.

Alternatively, conjugation of nucleotides to protein carriers through the phosphate terminus has recently been reported by Johnston et al. (1983). Apart from the advantages of the periodate oxidation method, the imidazolide method does not result in cleavage of the ribose ring and therefore, the hapten may better resemble the steric conditions present when the nucleotide is contained in a polymeric unit. As illustrated in Figure 15, the imidazolide method is initiated by electrophilic attack of carbonyldiimidazole on the 5'-phosphate group of the anhydrous nucleotide which results in the formation of an imidazole-nucleotide derivative with concomitant release of imidazole. Electrophilic displacement at the phosphate group of the imidazole-nucleotide derivative by the primary amino groups of the carrier protein leads to the formation of a protein-hapten conjugate designated as protein-Im (hapten) with the release of imidazole and carbon dioxide. One potential problem in this reaction is the requirement of an anhydrous environment during synthesis. The inability to maintain such an environment may be reflected by a low molar substitution ratio.

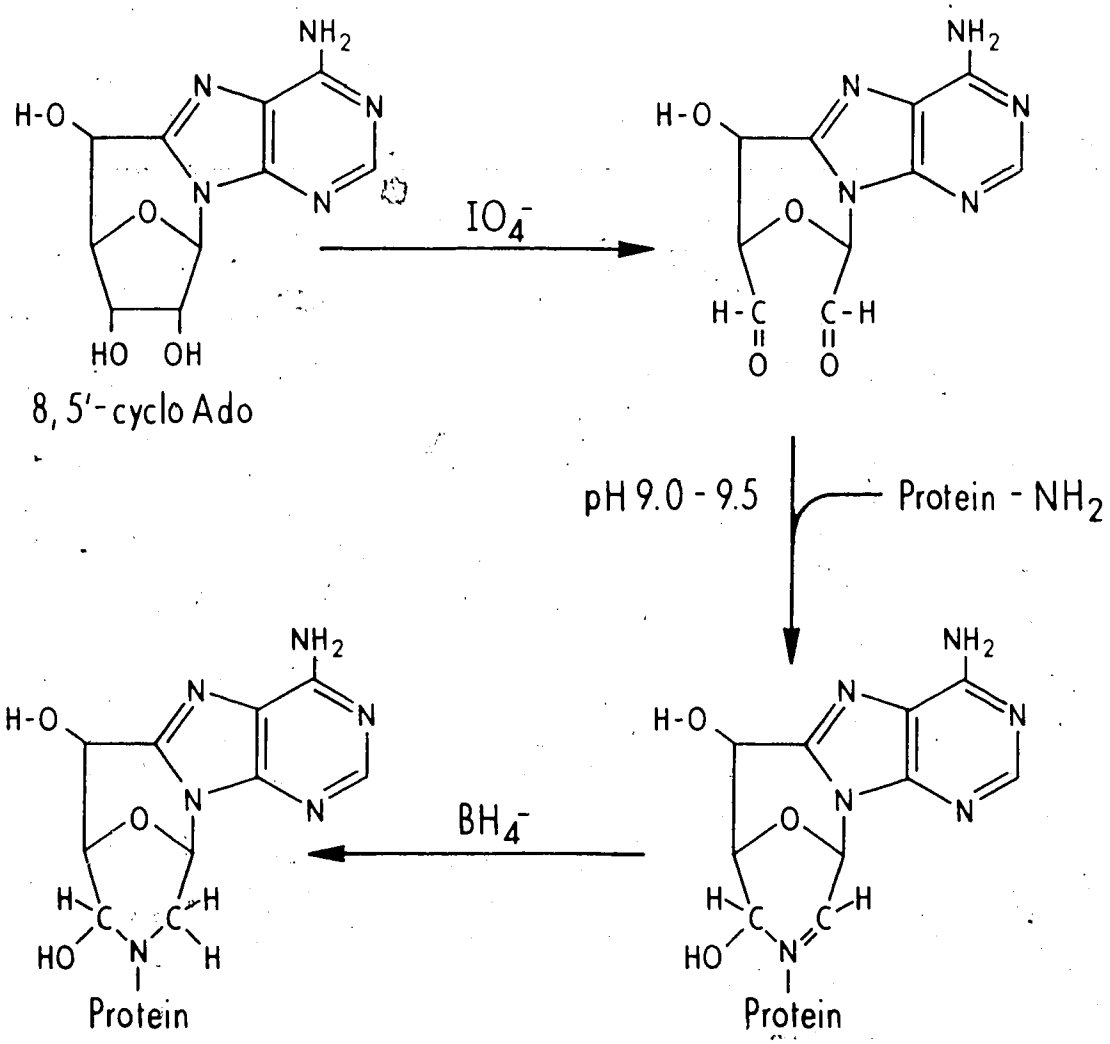

FIGURE 14: Schematic diagram illustrating the conjugation of 8,5'-cycloAdo to a protein carrier by the periodate oxidation method. (Adapted from Erlanger and Beiser, 1964).

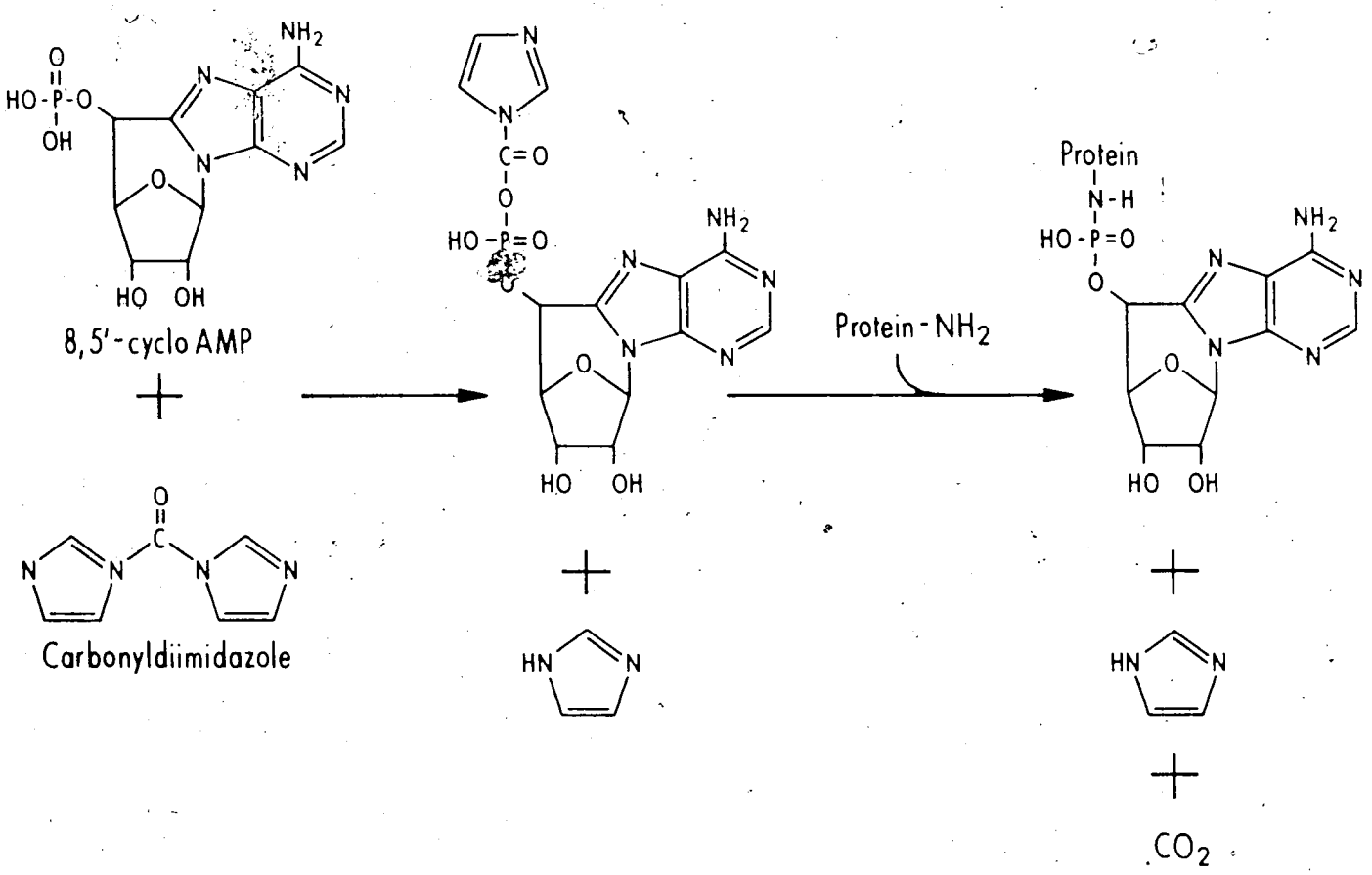

FIGURE 15: Schematic diagram illustrating the conjugation of 8,5'-cycloAMP to a protein carrier by the imidazolide method.

Characterization of both the reactants (hapten and carrier protein) and product (protein-hapten conjugate) by spectral analysis is a necessary exercise which serves two purposes. Firstly, such analysis provides the necessary information to determine the degree of hapten conjugation to the protein which, in turn, provides some assurances as to the reliability of the antibody titer. Munns and Liszewski (1980) suggest that immunogens containing less than 10 nucleosides per albumin molecule are usually poor immunogens, i.e. would lead to less than 100 µg of antibody per milliliter of antiserum). Secondly, spectral analysis may also reveal if the hapten has become altered during the conjugation reaction, thereby presenting different antigenic determinants to the antibodies as compared to the free hapten.

2. Induction of Antibodies

If immunochemistry is an art, then the immunochemist's artistic abilities are manifested during the immunization protocol. The framework pertaining to dosage and duration has been established in model systems and shall be reviewed. However, an immunoassay which utilizes polyclonal antiserum has a broad spectrum of antibodies with different qualities. Antibody heterogeneity from an individual test animal may exist at four levels as summarized by Steward and Steensgaard (1983):

1) Antibodies may belong to different classes or subclasses, involving structural and functional differences.
2) Within a single antibody subclass, antibodies exist against different antigenic determinants.
3) Antibodies against an individual antigenic determinant and belonging to the same subclass still consist of different antibodies showing affinity heterogeneity.

4) Individual antibodies may possess different physical properties beyond those defined by the subclass definition (i.e. solubility, cross-reactivity, conformational changes).

Eisen and Siskind (1964) demonstrated that antiserum from individual rabbits possessed average association constants which differed as much as 10,000 fold for 2,4-dinitrophenol when assayed by fluorescence quenching. In retrospect, this diversity in antibody quality is probably a conservative estimate since the purification methods employed by these authors selected against antibodies of low affinity (less than 10^5 litre - $mole^{-1}$) and cannot discriminate between association constants exceeding 10^9 litre - $mole^{-1}$. Protocols employing small quantities of immunogen (i.e. 5 mg as compared to 50, 100 or 200 mg) produced antibodies with higher average affinities and of which the affinity for the immunogen increased as the time interval between the immunizing injection and bleeding was prolonged (Eisen and Siskind, 1964; Siskind et al., 1967; Werblin et al., 1973; Steward and Steensgaard, 1983). Werblin et al. (1973) reported that a significant amount of the highest affinity antibody that a given rabbit would synthesize at any time during the immune response was already present by day 42. The theories of antibody affinity maturation are reviewed most recently in Steward and Steensgaard (1983).

The use of adjuvant during immunization has been a useful method to stimulate immune reactivity. Emulsions of Freund's Complete adjuvant (which contains Mycobacterium) with immunogens has been demonstrated to increase the inflammatory response in regional nodes, thereby amplifying the immune response and helping to stimulate the eventual formation of high affinity antibodies. Freund's adjuvant also partially protects

certain labile antigens by increasing their biological half-life permitting fewer subsequent inoculations.

3. Development of an Immunoassay

The types of immunoassays used to characterize anti-nucleotide antibodies include quantitative precipitation analysis, complement fixation, passive hemagglutination, hapten-conjugated bacteriophage inhibition, radioimmunoassay (RIA) and enzyme immunoassay (EIA). Excellent reviews which detail these techniques are available in the literature (Landsteiner, 1945; Schurrs and van Weeman, 1977; Munns and Liszewski, 1980; van Vunakis, 1980). Due to their high sensitivity and their potential for automation, radioimmunoassays have been the predominant assay to assess antisera during the past few decades. However, within the past decade, enzyme immunoassays have been recognized by many laboratories to have advantages compared with RIAs. Criticism of the EIA methodology is directed towards the use of radioisotopes, which, due to the short half-life of the most common isotopes used, limits the shelf life of the reagents. Furthermore, the reliance on gamma-emitting isotopes subjects the user to a potential radiation hazard. Enzyme immunoassays utilize enzymes to amplify the "signal" and thereby offer gains in sensitivity. The limit of this amplification factor is the ability to separate "signal" (generated by the specifically bound enzyme-labelled antibody complex) from the "noise" (consequent upon nonspecific labelled antibody binding) (Elkins, 1980). The EIAs may be divided into: 1) homogenous EIAs, more commonly called the enzyme-multiplied immunoassay technique (EMIT) (Rubenstein et al., 1972; Scharpe et al., 1976; Wisdom, 1976) and 2) heterogenous EIAs which

require separation of free antigen and antibodies from bound antigen-antibody complexes. Of the latter type of EIA, the most common technique has been the enzyme-linked immunosorbent assay (ELISA) which utilizes plastic surfaces to adsorb antibody-antigen complexes to effect separation from unbound material (Engvall and Perlmann, 1971; van Weemen and Schuurs, 1971). ELISA methodology and techniques have been the subject of symposia proceedings (Feldmann et al., 1976; Sever and Madden, 1977; Pa' 1978), reviews (Scharpe et al., 1976; Voller et al., 1976; Wisdom, 1976; Schuurs and van Weemen, 1977; Engvall, 1977; Voller et al., 1978; Engvall, 1980) and monographs (Engvall and Pesce, 1978; Voller et al., 1976; Maggio, 1980).

The early literature had suggested that ELISA assays were less or equal in sensitivity to RIAs (Dray et al., 1975; Belanger et al., 1976; Miyai et al., 1976; Jarvis, 1976). However, recent advances in methodology have demonstrated that the ultimate limit of sensitivity of EIA systems is determined by the nature of the antibody - antigen interactions. Although the affinity of the antiserum is the most important factor among the variables that determine the sensitivity of the ELISA, a number of parameters must be optimized to gain the highest sensitivity.

These are:

1) Concentration of antigen included in the coating buffer to adsorb on to the solid phase.
2) Times and temperatures for sample incubation.
3) Use of the lowest concentrations of antigen and antibody with longer times for substrate development.

4) Selection of enzyme-substrates which allow a greater amplification factor for specifically bound label.

In terms of the selection of appropriate enzyme - substrate combinations, no gross differences in a group of colorimetric ELISAs were observed (Schuurs and van Weemen, 1977). The sensitivity of ELISAs has surpassed conventional RIAs by up to 100 fold with the use of either a fluorogenic substrate (Kato et al., 1975 a,b; Kato et al., 1976; Hamaguchi et al., 1976; Yolken and Stopa, 1979; Shalev et al., 1980; Yolken and Leister, 1982) or a radiolabelled substrate in assays commonly described as USERIAs (van der Waart and Schuurs, 1976; Harris et al., 1979; Hsu et al., 1981). Therefore, the ELISA techniques have proven to be more sensitive than conventional RIAs to the point where antigen - antibody affinity may limit the assay.

The methodology that shall be employed in this study is the "Indirect" measurement of antigen as diagrammed in Figure 16 in which the antigen is used to coat the wells of polyvinyl chloride microtiter plates. Degradation of a chromogenic substrate by an enzyme yields an amplification factor which enables accurate and sensitive detection of the presence of enzyme.

Immobilization of an antigen to effect a rapid, facile separation of "free" antigen and antibodies from antigen - antibody complexes is characteristic of ELISAs. The most common technique, and that which is part of the success of ELISA methodology, is the passive, physical adsorption of antigens and antibodies to plastic carriers (i.e. polystyrene, polypropylene, polycarbonate) (Voller et al., 1976). The use of disposable polystyrene or polyvinyl chloride microtiter plates (Voller

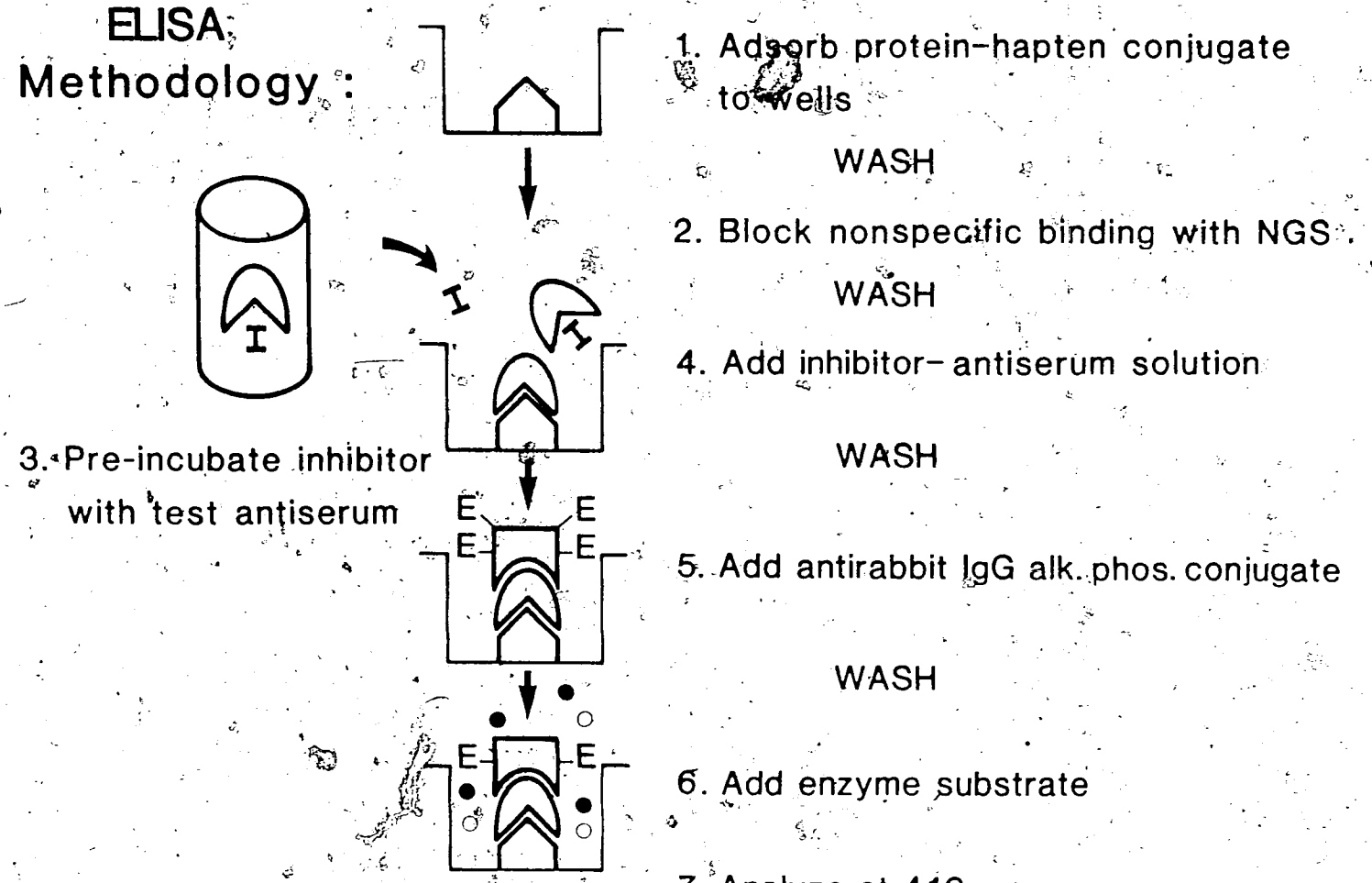

FIGURE 16: Schematic diagram illustrating the Indirect ELISA for assay of antigen.

et al., 1978) and tubes, (Engvall, 1971), has followed from this observation. The adsorption of proteins to plastic surfaces is a result of hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix. The rate and extent of coating is dependent upon: 1) the diffusion coefficient of the adsorbing material; 2) the ratio of the surface area to be coated to the volume of coating solution; 3) the concentration of the adsorbing substances; 4) the temperature and 5) the duration of the adsorption reaction (Engvall, 1980). The shortcomings of the physical adsorption of proteins to plastic surfaces are: 1) due to the loss of adsorbed protein, which may be up to 30% for the duration of the assay, the precision of the ELISA is adversely affected; 2) the plastic surfaces have a limited capacity for adsorption and 3) the adsorption process is nonspecific. This latter problem may arise in subsequent steps during the ELISA. For instance, during the incubation of the immobilized antigen with immunized serum, and during the incubation of the antigen - antibody complex with enzyme-labelled anti-IgG antibodies, two reactions may occur: 1) specific binding of the antigen with its corresponding antibody and 2) nonspecific adsorption of the antibody directly onto the solid phase. However, nonspecific adsorption may be minimized by the inclusion of a nonionic detergent such as Tween-20 in the buffers which prevents the formation of new hydrophobic interactions between added proteins and the solid phase without disrupting, to any appreciable extent, those hydrophobic bonds already present. Nonspecific binding of the enzyme-labelled antibody may be prevented by coating unbound sites on the solid phase with serum from a non-immunized animal of the same species as the enzyme-labelled antibody conjugate.

Variability in the adsorption process during an ELISA is probably the major factor in determining the precision of solid phase immunoassays (Denmark and Chessum, 1978; Herrmann and Collins, 1976). However, the drawbacks of passive adsorption are partially compensated by the ease and rapidity of separation of antibody - antigen complexes from free antigen and antibody. Many reports suggest that the optimal concentration of protein in the coating buffer is generally between 1 - 10 μg/ml (Engvall, 1980). Higher concentrations lead to increased adsorption, but two general problems become limiting: 1) the percentage of protein adsorbed decreases and 2) there is an increased desorption during the course of the ELISA assay probably related to layering of proteins.

The low yield of 8,5'-cycloAMP expected in irradiated DNA demands the use of a highly sensitive and specific assay. Therefore, ELISA methodology, which has the potential to be more sensitive than conventional RIAs due to enzyme amplification, has been selected for the development of an assay for 8,5'-cycloAMP.

MATERIALS AND METHODS

I. REAGENTS

Bovine serum albumin (BSA) Fraction V, Limulus polyhemus Hemolymph Type VIII (hemocyanin), alkaline phosphatase (1.1 U/mg), Sigma 104™ Phosphate Substrate (p-nitrophenol phosphate) and polyoxyethylene monolaurate sorbitan (Tween 20) were purchased from the Sigma Chemical Co.; polyvinyl chloride 96 well microtiter plates were purchased from Dynatech Laboratories Inc.; fetal calf serum, goat serum and Freund's adjuvants were purchased from Gibco Laboratories Inc.; goat anti-rabbit IgG alkaline phosphatase conjugate is a product of Miles Laboratories

Inc.; activated Type 5A 8-12 mesh molecular sieves were purchased from the J.T. Baker Chemical Co.; Spectra/por1 Dialysis membranes (MWCO 6,000 - 8,000) are a product of Spectrum Medical Ind. Inc. and diethanolamine is a Fisher Chemical Co. product.

II. PREPARATION OF PROTEIN-HAPTEN CONJUGATES

A. Preparation of Hemocyanin-Im (R) 8,5'-cycloAMP

(R) 8,5'-CycloAMP (13.8 mg, 38.2 μmol) was dried in vacuo with three consecutive additions and evaporations of 5.0 mL of dry pyridine followed by three consecutive additions and evaporations of 5.0 mL of dry benzene in a 50 mL conical flask. The residue was dissolved in a mixture of dry dimethyl sulfoxide (DMSO) (2.0 mL), triethylamine (100 μL, 720 μmol) and tri-n-octylamine (50 μL, 114 μmol) and transferred to a 13 x 100 mm borosilicate test tube. Carbonyldiimidazole (30 mg, 191 μmol) was added, and the reaction mixture was stirred for 60 min at room temperature. This solution was added to a solution of NaI (57.3 mg, 382 μmol) in 5.0 mL of dry acetone in a 50 mL centrifuge tube. The resultant white precipitate was vortexed, pelleted by centrifugation at 1500 rpm for 5.0 min then washed twice more with dry acetone in a similar manner. The imidazolide of (R) 8,5'-cycloAMP [Im(R) 8,5'-cycloAMP] was evaporated to dryness and then dried over P_2O_5 for 2 h before further reaction. The yield of Im (R) 8,5'-cycloAMP was 58% (10.2 mg, 23.2 μmol).

A solution of hemocyanin (50.0 mg, 16.7 nmol) in dry dimethyl sulfoxide (4.0 mL) was added to 10.2 mg (23.2 μmol) of Im (R) 8,5'-cycloAMP and stirred for 60 min in a 70°C water bath. The solution was placed in a dialysis membrane and dialyzed against 8.0 L (8 changes of 1.0 L) of PBS (pH 7.4) over a period of three days. Direct estimates of

the optical absorbance of the conjugate were not made since the conjugate precipitated. The conjugate was diluted to 10.0 ml with PBS (pH 7.4). Aliquots of 0.5 ml of this mixture were dispensed into tubes and stored frozen at -17°C.

B. Preparation of BSA-Im (S) 8,5'-cycloAMP

(S) 8,5'-CycloAMP (30.0 mg, 10 µmol) was dried in vacuo with three consecutive additions and evaporations of 5.0 ml of dry pyridine followed by three consecutive additions and evaporations of 5.0 ml of dry benzene in a 50 ml conical flask. The residue was dissolved in a mixture of dry dimethyl sulfoxide (2.0 ml), triethylamine (100 µl, 720 µmol) and tri-n-octylamine (50 µl, 114 µmol) and transferred to a 13 x 100 mm borosilicate test tube. Carbonyldiimidazole (80.4 mg, 550 µmol) was added and the reaction mixture was stirred for 60 minutes at room temperature. This solution was added to a solution of NaI (165 mg, 1.1 mmol) in 5.0 mL of dry acetone in a 50 mL centrifuge tube. The resultant white precipitate was vortexed, pelleted by centrifugation at 1500 rpm for 5.0 min, then washed twice more with dry acetone in a similar manner. The imidazolide of (S) 8,5'-cycloAMP [Im (S) 8,5'-cycloAMP] was evaporated to dryness and then dried over P_2O_5 for 2 h before further reaction. The yield of Im (S) 8,5'-cycloAMP was 61% (22.0 mg, 63.7 µmol).

A solution of BSA (50.8 mg, 736 nmol) in dry dimethylsulfoxide (4.0 mL) was added to 22.0 mg (63.7 µmol) of Im (S) 8,5'-cycloAMP and stirred for 60 min in a 70°C water bath. The solution was placed in a dialysis membrane and dialyzed with 8.0 L (8 changes of 1.0 L) of PBS (pH 7.4) over a period of three days.

The molar ratio may be determined spectrophotometrically by two methods since the protein-hapten conjugate was soluble in PBS (pH 7.4). The number of (S) 8,5'-cycloAMP (N) molecules bound per molecule of BSA, n(moles/mole) may be calculated from $A_{266\ nm}/A_{280\ nm}$ according to the following formula, assuming that the extinction coefficients of each component were not altered by conjugation:

$$n = \frac{\varepsilon_{BSA}^{280}\left(A_{266}/A_{280}\right) - \varepsilon_{BSA}^{266}}{\varepsilon_{N}^{266} - \varepsilon_{N}^{280}\left(A_{266}/A_{280}\right)}$$

where A_{266}/A_{280} is the ratio of the observed absorbance at 266 nm to that at 280 nm of the conjugated materials: $\varepsilon_{BSA}^{280} = 4.3 \times 10^4$ and $\varepsilon_{BSA}^{266} = 2.6 \times 10^4$, the molar extinction coefficients of BSA at 280 nm and 266 nm respectively, and $\varepsilon_{N}^{280} = 5.3 \times 10^3$ and $\varepsilon_{N}^{266} = 1.4 \times 10^4$, the molar extinction coefficients of (S) 8,5'-cycloAMP at 280 nm and 266 nm respectively. The molar substitution ratio of the conjugate and BSA was calculated in this manner from the UV spectra illustrated in Figure 17 ($A_{266}/A_{280} = 1.05$). The number of moles of (S) 8,5'-cycloAMP bound per mole of BSA was 2.3. The molar ratio may also be computed directly from the UV spectra (Figure 17) by calculating the difference spectrum between the spectra of the conjugate and BSA only. This analysis suggests that 1.6 moles of (S) 8,5'-cycloAMP are bound per mole of BSA. The conjugate was diluted to 10 mL with PBS (pH 7.4). Aliquots of 0.5 mL were dispensed into tubes and stored frozen at -17°C.

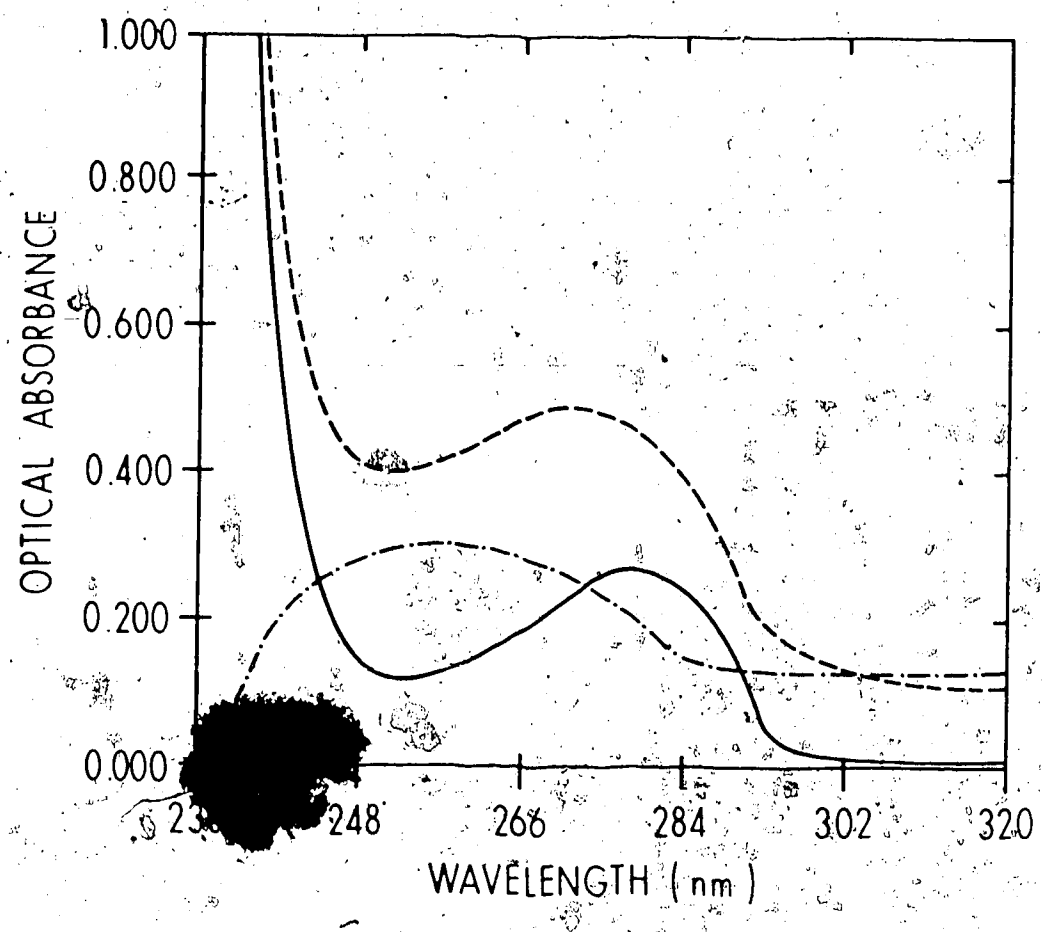

FIGURE 17: UV Spectra for conjugation of (S) 8,5'-cycloAMP to BSA by the imidazolide method. The molar substitution ratio (n) may be calculated from the difference spectrum (—·—) which is obtained by subtracting the absorbance spectrum of BSA (——) from the absorbance spectrum of BSA-Im (S) 8,5'-cycloAMP (— —).

C. Preparation of BSA-(S) 8,5'-cycloadenosine

(S) 8,5'-Cycloadenosine was formed by the dephosphorylation of the parent nucleotide. (S) 8,5'-CycloAMP 42.0 mg (122 µmol) was dissolved in 5.0 mL of Sørensen's glycine buffer II (pH 10.4) containing 10.0 mg of alkaline phosphatase and incubated for 18 h at 37°C. Completion of the dephosphorylation was assessed by following the loss of (S) 8,5'-cycloAMP by HPLC in the supernatant. Crystals of (S) 8,5'-cycloAdo

precipitated from solution and were collected by centrifugation at 1000 rpm for 2 min. The crystals were washed twice with deionized water. The yield of (S) 8,5'-cycloAdo exceeded 95%.

Conjugation of (S) 8,5'-cycloadenosine to BSA was performed by the periodate oxidation method (Erlanger and Beiser, 1964). (S) 8,5'-Cycloadenosine (32.0 mg; 121 µmol) was dissolved in 2.0 mL of 0.1 M $NaIO_4$ and allowed to stand for 20 minutes at ambient temperature. Excess IO^-_4 was decomposed by the addition of 0.1 mL of 1.0 M ethylene glycol during a 5 min incubation at ambient temperature. The reaction mixture was then added with stirring to 3.0 mL of an aqueous solution (adjusted to pH 9 - 9.5 with 5% $NaCO_3$) containing 95 mg (1.38 µmol) of bovine serum albumin. Stirring was continued for 60 min and the pH was maintained at 9 - 9.5 with 1.0 N NaOH. At the end of this period, a solution containing 45.0 mg (1.19 mmol) of $NaBH_4$ in 0.5 mL of deionized H_2O was added slowly and the reaction mixture was stirred for 18 h. One mL of 1.0 N HCOOH was added, followed one hour later by the addition of 1.0 N NH_4OH to bring the pH to 8 - 8.5. The solution was placed in a dialysis membrane and dialyzed against PBS (pH 7.3) over the next 72 h with 12 x 1.0 L changes of buffer. The final volume of the dialysate was brought to 15.0 mL with PBS (pH 7.3). Aliquots of 0.5 mL were pipetted into 0.5 mL tubes and stored below -17°C until use.

The extent of substitution of (S) 8,5'-cycloAdo onto BSA was determined spectrophotometrically by calculating the difference spectrum between the conjugate and BSA (Figure 18). By this method, 5.4 molecules of (S) 8,5'-cycloAdo were conjugated to one molecule of BSA. The molar substitution ratio calculated from the spectrum of BSA-(S) 8,5'-cycloAdo ($A_{266/280}$ = 0.83) was 1.0.

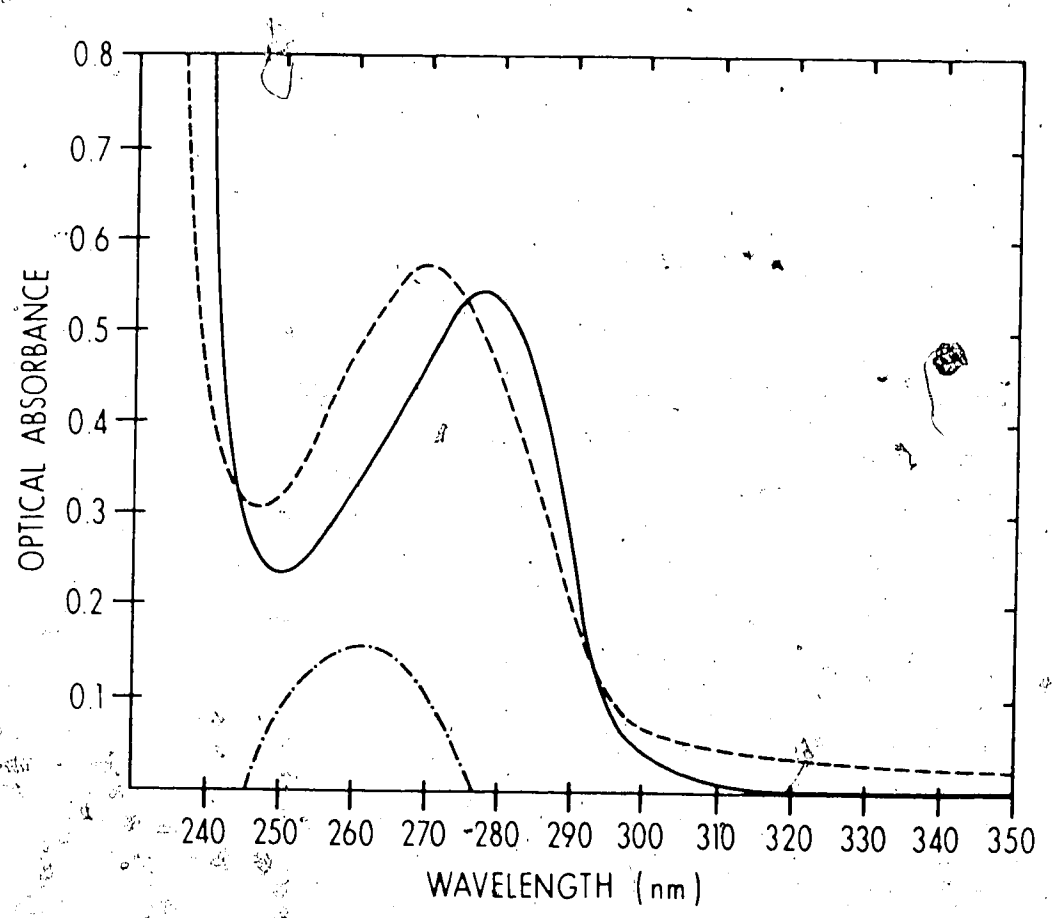

FIGURE 18: UV Spectra for conjugation of (S) 8,5'-cycloAdo to BSA by the periodate oxidation method. The molar substitution ratio (n) may be calculated from the difference spectrum (·—·) which is obtained by subtracting the absorbance spectrum of BSA (——) from the absorbance spectrum of BSA-(S) 8,5'-cycloAdo. (— —).

III. IMMUNIZATION AND TREATMENT OF ANTISERA

Two New Zealand white female rabbits R001 and R002 were injected with the hemocyanin-Im (R) 8,5'-cycloAMP conjugate. Prior to the initial immunization, 30 mL of blood were collected from each rabbit by cardiac puncture using an 18 gauge needle and 50 mL syringe to act as control sera in subsequent assays. Each rabbit received 5.0 mg of conjugate in 0.5 mL of PBS (pH 7.4 emulsified in 0.5 mL of Freund's Complete

adjuvant injected at multiple subscapular sites. Subsequent injections of 5.0 mg of conjugate in 0.5 ml PBS (pH 7.4) emulsified in Freund's Incomplete adjuvant were administered by the same route on days 21, 46 and 91. Sera (30 ml) were collected by cardiac puncture on days 21 ("ra" bleed), 43 ("rb" bleed), 60 ("rc" bleed) and 80 ("rd" bleed).

Two New Zealand white female rabbits S003 and S004 were injected with the BSA-Im (S) 8,5'-cycloAMP conjugate. Prior to the initial immunization, 30 ml of blood were collected by cardiac puncture from each rabbit. Each rabbit received 5.0 mg of conjugate as above. Subsequent boosts of conjugate in Freund's Incomplete adjuvant were administered on days 21 and 42. Sera were collected on days 35 ("sa" bleed) and 50 ("sb" bleed).

Two New Zealand white female rabbits A1 and A2 were injected with the BSA-(S) 8,5a-cycloAdo conjugate. Each rabbit received 6.3 of conjugate as above. Subsequent boosts of conjugate in Freund's Incomplete adjuvant were administered on days 13, 28, 41, 55 and 70. Sera were collected on days 90 ("Aa" bleed), 126 ("Ab" bleed) and 160 ("Ac" bleed).

Immediately following collection of blood by cardiac puncture, the blood was allowed to clot for one hour at 37°C, the clot was removed and the serum was centrifuged at 2,000 rpm for 10 minutes at 4°C. The supernatant was drawn off and the serum was recentrifuged twice. Aliquots of 2.0 mL were stored at -17°C. Repeated thawing and refreezing were avoided.

IV. ELISA METHODOLOGY

A. Determining the Optimal Dilution of Reagents

The reagent dilution assay is designed to determine the optimal

dilution of conjugate used to coat the wells of the microtiter plates, the titer of the antisera, and the dilution at which an ELISA reading of 50% maximum value occurs. This screening assay was performed for each bleed prior to inhibition assays. Negative controls for this screening assay and competitive inhibition assays consisted of: 1) no protein-hapten conjugate in all of the wells in the first row; 2) 0.5 mg/mL BSA in all the wells in the second row and 3) 10^{-2} dilutions of serum collected before the initial immunization in columns one and two. Rows three to eight of the plates were coated with a 0.2 ml aliquot of protein-hapten conjugate serially diluted in 60 mM coating buffer (pH 9.8) for 14 h at 4°C. The wells of the plates were each washed five times in PBS-T (pH 7.4) with a plastic wash bottle. To prevent non-specific binding of the goat anti-rabbit IgG alkaline phosphatase conjugated antiserum to the wells, each well was exposed to 0.2 ml of 1% goat serum in PBS-T for 1.5 h at 37°C and then washed five times with PBS-T. Serum obtained from the same rabbit before and after immunization was serially diluted in PBS-T and 100 µl of the diluted serum were added to each well. After a 2 h incubation at 37°C, the plates were washed five times with PBS-T and incubated with 0.1 mL of goat anti-rabbit IgG alkaline phosphatase conjugate per well (1:400 dilution in PBS-T) supplemented with 1% fetal calf serum to prevent nonspecific binding. The plates were washed five more times with PBS-T and then twice with a 0.1 M diethanolamine buffer (pH 9.8) before adding 0.1 mL/well of Sigma 104™ Phosphatase Substrate (0.1 mg/mL) diluted in 0.1 M diethanolamine buffer. The enzymatic reaction was allowed to continue for 30-45 minutes then inhibited with 50 µL/well of 3.0 N NaOH. The extent of the enzymatic reaction was measured using a Dynatech MR600 Microplate Reader

(Dynatech Laboratories Inc.) operating with a reference wavelength of 610 nm and an operating wavelength of 410 nm. The spectrophotometer was blanked on an uncoated well containing a 10^{-2} dilution of preimmune rabbit serum.

B. Developing an Inhibition Curve

The optimal dilutions of antigen coating the ELISA wells and of positive antiserum were determined in Section IV-A. For inhibition studies, the dilution of antiserum corresponding to 50% of the maximal ELISA reading from the dilution assay was selected. In a representative experiment, a serial dilution of a potential inhibitor was prepared. A 180 µL aliquot of each concentration of inhibitor was added to 20 µL of antiserum diluted to 10^{-1}. Preimmune serum was diluted to a final dilution corresponding to that of the positive antiserum. These solutions were incubated for 2 h at 37°C prior to addition to the microtiter plates. Exposure of these solutions to the microtiter plates was for 2 h at 37°C, following which the inhibitor solution was carefully washed from the wells with a Titertek 8 channel pipettor and five changes of PBS-T. Addition of the anti-rabbit alkaline phosphatase second antibody and subsequent exposure of the enzyme substrate to the wells was described in Section IV-A.

V. EFFECT OF INCREASING CONCENTRATIONS OF 5'-AMP ON THE SPECIFICITY OF THE ANTISERUM

Competitive inhibition studies were performed as described in Section IV-B using the (R) and (S) diastereoisomers of 8,5'-cycloAMP in the presence of 16.6, 2.27 and 0.69 µM solutions of 5'-AMP prepared in PBS-Tween (pH 7.3). The reagent antiserum was from "rc" bleed.

VI. DOSE-YIELD RESPONSE FOR THE FORMATION OF 8,5'-cycloAMP FROM IRRADIATED SOLUTIONS OF 5'-AMP

To investigate the formation of 8,5'-cycloAMP in irradiated solutions of 5'-AMP, 174 mg (501 μmol) of 5'-AMP were added to 90 ml of deionized H_2O, the pH of the solution was adjusted to 8.90 with NaOH and the total volume was brought to 100 ml with deionized water. Aliquots of 2.0 ml of this solution were added to each of 30 - 13 x 100 mm borosilicate glass test tubes. Samples were saturated with nitrous oxide (see Chapter 1: Methods) for 15 min prior to and during irradiation. Samples were irradiated in triplicate at 70.8 Gy/min in a lucite holder (Plate 1) in the dose range of 0 - 5000 Gy. Following irradiation, the volume of the samples was returned to 2.0 ml with deionized water and divided into two 1.0 ml portions. One series of irradiated solutions received no further treatment. The second series of irradiated solutions was hydrolyzed with 0.3 N HCl for 1.0 h in a 100°C water bath. Following hydrolysis the volume was adjusted to 1.0 mL. Both series of hydrolyzed and unhydrolyzed samples were diluted 1000-fold in PBS-I (pH 7.3) prior to competitive inhibition analysis. Calibration curves in which the molar concentration of inhibitor was plotted as a function of the ELISA value (B/[illegible]) were completed on each microtiter plate and the concentration of [illegible] measured from these curves. The reagent antiserum was obtained from "rc" bleed.

RESULTS

I. ANTIBODIES RAISED TO BSA-(S) 8,5'-cycloAdo

A. Reagent Dilution Assay

Antiserum from rabbit A2 immunized with this conjugate ("Aa" bleed) was screened using the same conjugate coated onto the wells of microtiter plates. The optimal dilution of BSA-(S) 8,5'-cycloAdo for coating the polyvinyl chloride microtiter plates was 1 x 10^{-2}, corresponding to a conjugate concentration of approximately 63 µg/ml (Figure 19). Reducing the conjugate concentration 10-fold resulted in a significant decrease in the ELISA reading. Plates coated with a similar concentration of BSA showed no significant cross-reactivity. For the assay, 1% BSA was included in the serially diluted antiserum preparations to adsorb antibodies which are directed to antigenic determinants

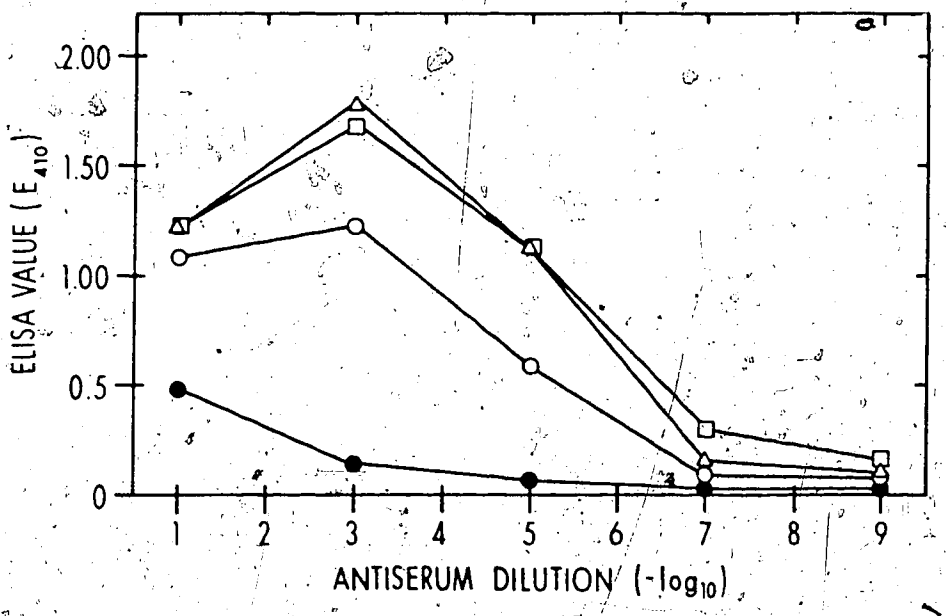

FIGURE 19: Reagent dilution assay for anti-BSA-(S) 8,5'-cycloAdo antiserum. Antiserum containing anti-BSA-(S) 8,5'-cycloAdo antibodies was serially diluted, adsorbed to microplate wells and screened with the ELISA procedure as described in the text. (Dilution of conjugate in coating buffer: △ 10^{-1}, □ 10^{-2}, ○ 10^{-3}, and ● 10^{-4}.)