Hybridization of ^{125}I-Labeled Ribonucleic Acid†

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ABSTRACT: A detailed characterization of the iodination of RNA using ^{125}I is described. A modification of the Commerford technique allows specific activities in excess of 3×10^7 cpm/μg to be obtained routinely. The iodinated RNA retains its specificity in DNA–RNA hybridization with only a negligible decrease in the stability of DNA–RNA hybrids.

Several recent advances in experimental methodology have facilitated the isolation of a number of specific mRNAs from eucaryotic cells (Suzuki and Brown, 1972; Palacios *et al.* 1973; O'Malley *et al.*, 1972; Aviv and Leder, 1972). However, it is not always possible to obtain high specific radioactivities by the administration of labeled precursors *in vivo*. Such high specific radioactivities are essential for DNA–RNA hybridization experiments designed to enumerate, or compare among related species, genes responsible for the synthesis of such messengers, particularly when this number is one or a few per haploid genome (Bishop *et al.*, 1972; Packman *et al.*, 1972; Suzuki *et al.*, 1972). It is possible to circumvent this difficulty by the enzymatic synthesis of a complementary DNA strand using RNA-dependent DNA polymerase (Aviv and Leder, 1972). Although specific radioactivities exceeding 10^7 cpm/μg can be achieved, the RNA may not be copied uniformly (Taylor *et al.*, 1973) or with complete fidelity. Thus, for many purposes, including precise hybridization assays, *in vitro* labeling of RNA to high specific radioactivity is desirable.

Several methods for *in vitro* labeling of RNA have been described (Smith *et al.*, 1967; Lee and Gordon, 1971), but only iodination offers the possibility of specific radioactivity exceeding 10^6 cpm/μg. Commerford (1971) described a method for introducing ^{125}I into both DNA and RNA. However most of his studies involved DNA and only low specific radioactivities were actually obtained for RNA. Getz *et al.* (1972) have presented an adaptation of the method for RNA although again only 2.2×10^5 cpm/μg was attained. In the succeeding pages we present further modifications of the method whereby small quantities of RNA may be routinely labeled with ^{125}I to as much as 3×10^7 cpm/μg. Specific activities in the same range were recently reported by Scherberg and Refetoff (1973).

Materials and Methods

Chemicals. Carrier-free ^{125}I was obtained from Amersham/Searle (pH 8–11) and New England Nuclear Corp. (pH >12). Thallium chloride was obtained from K & K Laboratories. Solutions of $TlCl_3$ should be prepared immediately before use to avoid reduction of the thallic ion. Dilution below 10^{-3} M results in cloudiness, probably the formation of thallium hydroxide complexes. Accordingly stock solutions were prepared at 5×10^{-3} M and added directly to the reaction mixture. The nonionic detergent NP 40 was provided by the Shell Oil Co.

Isolation of DNA and RNA. DNA was isolated from decapitated 12-day chick embryos by minor modifications of the Marmur (1961) method. *Escherichia coli* RNA was isolated by standard procedures. Chicken RNA was extracted from 12-day chick embryos after first isolating polyribosomes from a postmitochondrial supernatant of tissue homogenized in NP 40. The polyribosomes were dissolved with 0.2 M LiCl–0.002 M sodium acetate–0.5% sodium dodecyl sulfate (pH 5.2) followed by two phenol extractions at room temperature (Shearer and McCarthy, 1967). The 28S component was isolated by sucrose gradient centrifugation, collected by ethanol precipitation, and stored in aqueous solution at −20°.

Iodination of RNA. Reactions were carried out in 1.5-ml stoppered siliconized specimen vials in a total volume of 30 μl. Concentrations of potassium iodide and thallic chloride were standardized at 7×10^{-5} and 8×10^{-4} M, respectively. The RNA concentration, ionic strength, temperature, and pH were varied as specified. All reactions were carried out for 15 min, and terminated by chilling to 0° and adding 200 μl of 0.5 M phosphate buffer (pH 6.8), containing 0.01 M mercaptoethanol. Unstable intermediates were destroyed by heating at 60° for 45 min (Commerford, 1971). RNA was separated from free iodide by means of a G-75 Sephadex column (0.9×19 cm) at room temperature equilibrated with 2 × SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.02% sodium azide (Figure 1).

^{125}I Counting. Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with a gain of 25% and window settings of 50–900 (Rhodes, 1965). Bray's solution was used for aqueous samples and a toluene Omnifluor solution was used for counting radioactivity on glass filters. A scintillation probe designed for ^{125}I detection (Wm. B. Johnson and Assoc. Inc., Montville, N. J.) was used to monitor working areas.

Determination of Specific Radioactivity. Small amounts of RNA were measured by the fluorescence method of Le Pecq and Paoletti (1966). Fluorescence was measured in a Turner Model 210 spectrofluorimeter using 28S RNA as a standard. Values for specific radioactivity obtained by this method agreed well with the results of calculations based on the amount of ^{125}I and RNA used and the fraction of the isotope incorporated.

DNA–RNA Hybridization. ^{125}I-Labeled RNA was hybridized to 11 μg of chick DNA immobilized on 6.4-mm diameter Millipore filters according to Gillespie and Spiegel-

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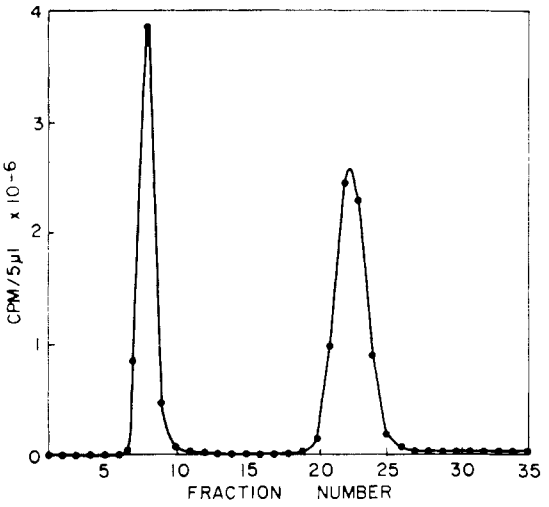

FIGURE 1: Sephadex G-75 chromatography of iodination mixture. 20 μg of 28S rRNA in a total volume of 30 μl was labeled with 1 mCi of ^{125}I at 70° with a pH 5.0 acetate buffer (0.05 M Na^+) and a total iodide concentration (^{125}I plus ^{126}I) of 4×10^{-5} M. 450-μl fractions were taken in the elution from G-75 Sephadex. 5-μl samples were counted in Bray's solution with peak samples diluted 100-fold before counting.

man (1965) in 0.1 ml of 2 × SSC at 70° or 5 × SSC and 50% formamide at 45° for 16 hr. The filters were washed once in 1 ml of reaction buffer at the incubation temperature and once at 37° in 2 × SSC. The filters were then incubated 45 min at 37° in 1 ml of 2 × SSC containing 40 μg of pancreatic RNase and 5 units of T_1 RNase. The filters were washed once more in 2 × SSC at 37°, blotted, and air-dried. Control filters containing bacteriophage T4 DNA were used with each reaction. Essentially all of the DNA remained on the filters under these reaction conditions as judged by recovery after DNase digestion of the filters after incubation. Hybrids formed in solution were assayed by treatment with the two ribonucleases as above described.

Results

Labeling Procedures. Some initial difficulties were encountered as a result of using small reaction volumes. High concentrations of small amounts of RNA are necessary to facilitate efficient incorporation of ^{125}I. Since radioiodide is often supplied at high pH, it was necessary to add a strong buffer to achieve a pH of 5 or below in the reaction solution. Under these conditions only small amounts of iodide were incorporated. This is probably due to the secondary or tertiary structure of the RNA at high ionic strength and iodide impurities in the reagents. This problem is readily obviated by using ^{125}I supplied at pH 8–11. In this case lower buffer concentrations suffice to control the pH.

According to Commerford (1971), a twofold increase in labeling of DNA results from each 10° rise in temperature with short labeling periods. The same is true for RNA at temperatures between 40 and 70° (Table I). Only a slight increase was obtained by raising the temperature to 80°. In all further experiments the reaction temperature was 70°.

As is the case for DNA, the efficiency of labeling decreases above pH 5 (Table I). Between pH 4 and 5 in buffers containing 0.05 M Na^+, no appreciable change was observed. Thus in order to minimize depurination and deamination, pH 5 was chosen as the optimum. The decrease in labeling efficiency with increasing ionic strength, is directly demonstrated by adding NaCl to the reaction mixture. Under these conditions incorporation is markedly reduced probably due to secondary or tertiary structure.

TABLE I: Influence of Reaction Conditions on Labeling.[a]

RNA (μg/ml)	Reaction Temp (°C)	pH	Ionic Strength (M)	Cytosine: Iodide	% Incorp
(a) 600	40	5.0	0.05	7.8	3
600	50	5.0	0.05	7.8	6
600	60	5.0	0.05	7.8	12
600	70	5.0	0.05	7.8	20
600	80	5.0	0.05	7.8	23
(b) 600	70	4.0	0.05	7.8	20
600	70	4.5	0.05	7.8	20
600	70	5.0	0.05	7.8	20
600	70	5.5	0.05	7.8	3
600	70	6.0	0.05	7.8	2
(c) 600	70	5.0	0.20	7.8	15
600	70	5.0	0.40	7.8	12
(d) 600	70	5.0	0.05	7.8	32
300	70	5.0	0.05	3.9	15
130	70	5.0	0.05	1.7	12
65	70	5.0	0.05	0.8	14
(e) 600	70	5.0	0.05	7.8	28
600	70	5.0	0.05	7.8	38
600	70	5.0	0.05	7.8	32

[a] Chicken 28S rRNA was incubated for 15 min at the designated temperature in 30 μl of a solution containing 6 μCi of ^{125}I, 7×10^{-5} M KI, and 8×10^{-4} M $TlCl_3$. Series (a) illustrates the effect of temperature, (b) the effect of pH, (c) that of ionic strength varied by addition of NaCl, and (d) the influence of RNA concentration. In series (e) the limits of reproducibility are assessed in triplicate reaction mixtures at optimum temperature, pH, and ionic strength. The buffer at pH 4 was sodium formate, at pH 4.5 and 5.0 sodium acetate and at pH 5.5 and 6.0 sodium maleate. The percentage incorporation of iodide into RNA was assessed by Sephadex G-75 gel exclusion chromatography (Figure 1). Series (a), (b), and (c) represent experiments carried out in parallel and the results are directly comparable. The same applies to series (d) and (e). The use of two different radioiodide solutions may account for variation between the two sets of results.

Effect of RNA Concentration. The effect of iodide concentration in labeling efficiency has been reported previously (Commerford, 1971; Getz *et al.*, 1972; Scherberg and Refetoff, 1973). Optimal concentrations appear to be in the range of 10^{-4}–10^{-5} M. This concentration range could be achieved by using 1–10 mCi of ^{125}I in 30 μl of reaction mixture. A detailed study was made of the efficiency of incorporation using 7×10^{-5} M iodide as a function of RNA concentration. The fraction of iodine incorporated remains relatively constant below 300 μg/ml thus resulting in proportionally higher specific radioactivities at low RNA concentration (Table Id). The study was not extended below 65 μg/ml since under these conditions some 16% of the cytosine residues become iodinated, a level which begins to affect hybridization efficiency and fidelity. However, no decrease in ribonuclease sensitivity or increased iodide lability was encountered.

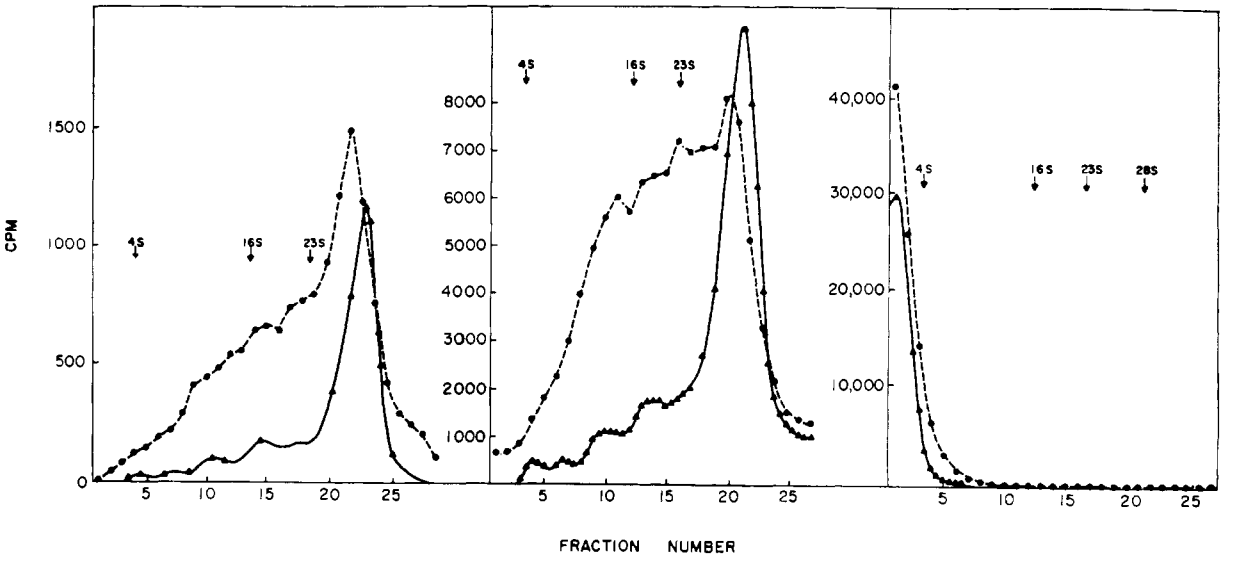

FIGURE 2: Sedimentation profiles of labeled and unlabeled 28S rRNA. 28S rRNA was sedimented through a 5 × 20% sucrose gradient at 40,000 rpm in a SW-40 Spinco rotor at 20° for 5.25 hr in 0.1 M NaCl–0.001 M EDTA–0.01 M Tris (pH 7.6). *E. coli* RNA was run on an identical gradient for additional standards. (——) Optical density of unlabeled RNA; (---) 100-μl samples of labeled RNA. (a) RNA specific activity, 60,000 cpm/μg; (b) RNA specific activity 3 × 10^7 cpm/μg; (c) the same high specific activity RNA pretreated with a mixture of T_1, 5 units; pancreatic ribonuclease, 40 μg/ml, at 37° for 45 min.

As a final check on the labeling methodology three identical reaction mixtures were used and the fractional incorporation determined (Table Ie). It is perhaps not surprising that variability is detectable since the reaction volume was so small.

Degradation of the RNA during the Labeling. The temperature and pH conditions required for labeling suggest that some degradation is inevitable. This was observed when 28S RNA was labeled to either a low or high specific radioactivity. Figure 2 illustrates sedimentation profiles of 28S RNA labeled to 6 × 10^4 and 3 × 10^7 cpm per μg. Degradation is evident in both with a suggestion of larger effects at higher specific radioactivity. However in both cases the data are consistent with an average of one break in about half of the molecules. Radiation damage does not appear to be a major problem since high specific activity RNA stored for 3 half-lives exhibited no decrease in mean molecular weight. The high specific radioactivity 28S RNA was completely sensitive to ribonuclease (Figure 2c).

Filter Hybridization of Iodinated RNA. Possible effects of hybridization characteristics were explored by incubating various quantities of high specific activity 28S chicken RNA with homologous DNA filters. Because of the limited amount of high specific activity 28S rRNA, and a background of 0.4% of input counts, saturating concentrations could not be used. The saturation value can nevertheless be obtained from a Scatchard plot as the intercept on the x axis (Figure 3). This estimate, approximately 11 genes/haploid genome, is slightly lower than the reported value in the literature (Merits *et al.*, 1966). The dissociation constant can also be determined from the slope of the linear plot ($K_d = -1/\text{slope}$). The value obtained was 3.8 × 10^{-7} mol of nucleotides/l which is very close to that reported for other DNA–RNA hybrids (Kennell, 1971; Bishop, 1970).

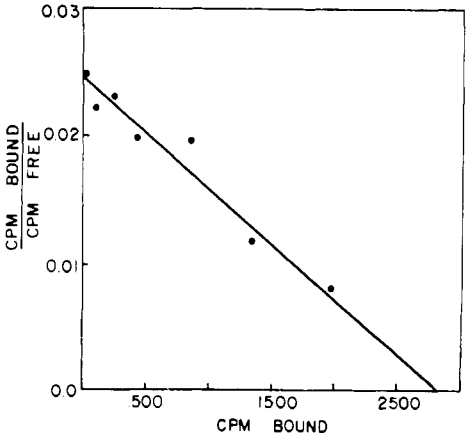

FIGURE 3: Scatchard plot of hybridization data. Hybridization of 28S rRNA, 1 × 10^7 cpm/μg, was performed in 2× SSC at 70° for 16 hr. The RNA–DNA ratio at saturation is 2.8 × 10^{-5} and K_d is 3.8 × 10^{-7} mol of nucleotides/l.

The fidelity of pairing in the DNA–RNA hybrids was assessed by determination of their thermal dissociation behavior. Radioactivity eluting from a T4 DNA control filter was subtracted from the radioactivity eluted from the chick filter (Figure 4). The filter duplexes exhibited a T_m of 86° in 0.5 × SSC. Assuming a G + C content of 64% (Merits *et al.*, 1966), native DNA should have a T_m of 91.5° under these conditions (Frank-Kamenetski, 1971). Since DNA–RNA hybrids typically melt approximately 4° lower than double-stranded DNA (Chamberlain and Berg, 1963), we conclude that the ^{125}I-labeled RNA–DNA hybrid melted about 1.5° lower than expected if perfectly matched. This would indicate between 1.4 and 2% mismatching (Laird *et al.*, 1969; Hutton and Wetmur, 1973; Ullman and McCarthy, 1973), which is within experimental error of the estimated 1% of the bases which are labeled with iodide. Thus the degree of mismatching observed can be attributed to the iodinated bases themselves rather than unknown damage introduced during the labeling procedure.

Solution Hybridization of Iodinated RNA. The hybridization behavior of ^{125}I-labeled chicken rRNA was also tested by solution reactions under conditions (Melli *et al.*, 1971) of DNA excess. Labeled RNA was incubated with chicken DNA at four different RNA–DNA ratios and the hybridization assayed by resistance to ribonuclease (Figure 5). Under these conditions DNA–DNA and RNA–DNA reactions are in competition and the extent of RNA–DNA hybrid formation

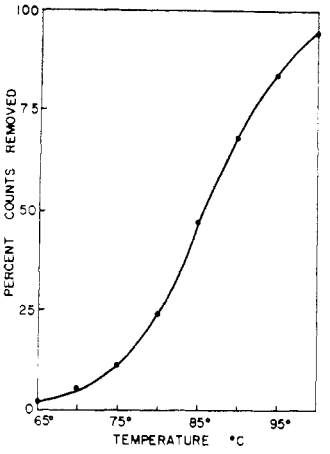

FIGURE 4: Melting curve of iodinated 28S RNA. A DNA filter containing 1300 cpm of hybridized 28S rRNA was incubated for 5 min in 0.5 ml of 0.5 × SSC at 5° intervals from 60 to 100°. The eluted radioactivity at each temperature was then counted in Bray's solution.

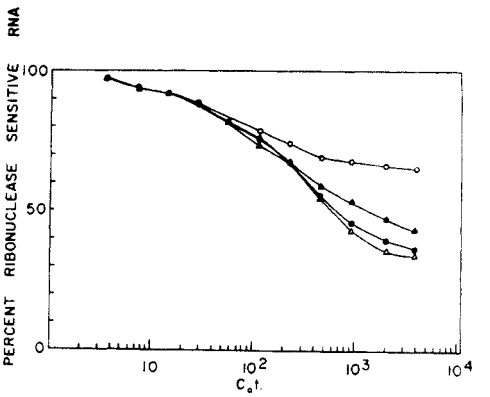

FIGURE 5: Reaction of ^{125}I 28S RNA with excess chicken DNA in solution. Hybridization reactions were performed in 50% formamide 5 × SSC at 45°. Sealed capillaries containing 0.02 ml of reaction mixture were removed at appropriate times and diluted with 1 ml of cold 2 × SSC containing 40 μg of pancreatic RNase and 5 units of T_1 RNase. The solutions were incubated at 37° for 45 min before trichloroacetic acid precipitation. DNA concentration was 4.0 mg/ml; RNA concentration was 2.9 ng/ml (△), 11.6 ng/ml (●), 43.8 ng/ml (▲), and 145 ng/ml (○). Specific activity of the RNA was 1.6×10^7 cpm/μg. Background was 3% of input counts.

is dependent on the RNA–DNA ratio. However under a constant set of conditions K_1, the rate constant for DNA–DNA duplex formation and k_2, the rate constant for RNA/DNA hybrid formation both have fixed, though unknown, values. Thus, with four sets of data where the DNA–RNA ratio is known, a computer program designed for best fit to the appropriate equation (Straus and Bonner, 1972) can be used to determine the three unknowns, k_1, k_2, and the number of genes. This approach obviates the necessity for external standards designed to determine rate constants. Using these principles, values of 3.6 for k_1/k_2 and 20–25 for the number of genes were obtained which fit the experimental data of Figure 5.

Discussion

With respect to the parameters investigated in Commerford's study and this investigation, it appears that the iodination of RNA and single-stranded DNA are quite similar. The yield of iodination *vs.* pH has been reinvestigated and shown to have a sharp dependence between 5.5 and 5.0 with a plateau below this value. This is consistent with Commerford's (1971) assessment that the pK_a of the intermediate 5-iodo-6-hydroxydihydrocytidylic acid was somewhat below 5.5.

Degradation of RNA and single-stranded DNA cannot be compared directly because of the slightly different reaction conditions employed. However, it is apparent that both nucleic acids are degraded only to a minor extent. It has been observed that slightly less degradation of RNA occurs at lower incubation temperatures. Thus if conservation of size is more important than specific activity, a reaction temperature of 50° is to be preferred.

The effect of RNA concentration on the efficiency of iodination was a little unexpected since it had been previously shown that reducing the iodide concentration at constant cytosine concentrations reduced the yield of incorporated iodide. One explanation could be the fact that only the final product is being looked at and not the immediate product of the second-order reaction. At the lower RNA concentrations a larger fraction of the intermediate could proceed to the final product due to the differences in relative concentrations of iodide and intermediate with different RNA concentrations. Because of the extensive iodination and difficulty of accurately determining specific activities on small amounts of RNA, it is advisable to use at least 4 μg of RNA if possible. The efficiency of labeling is clearly facilitated by using small reaction volumes. However, volumes much less than 30 μl may prove impracticable due to difficulties in assembling the components and poor recovery.

Iodination reduces the ability of cytosine to base pair (Commerford, 1971) perhaps due to a change in dipole moment. However this effect does not seriously impair the specificity or efficiency of hybridization unless a high percentage of cytosine is iodinated. With about 1% 5-iodocytosine, the thermal stability of chicken 28S rRNA hybrids is within 2° of that expected. A similar conclusion was arrived at by Getz *et al.* (1972) although any effects on T_m observable by them would have been superimposed on the large amounts of mismatching present in their hybrids of redundant DNA.

The iodinated RNA was used to measure the number of 28S rRNA genes in chicken DNA. Estimates made by two independent experimental procedures were in the range of 10–25 copies/haploid genome. These values are not due to biased iodination of nonbase-paired regions in the RNA since preliminary data have indicated that the cytosines are randomly iodinated when high temperatures and low ionic strengths are utilized. Although this degree of multiplicity seems low compared to accepted values for mammals and amphibia (Birnstiel *et al.*, 1971), it is in reasonable agreement with the earlier conclusion of Merits *et al.* (1966). Although they calculated 15–34 gene copies, the base composition of the hybridized RNA was not that of rRNA, suggesting the presence of impurities. The agreement between these three sets of data serves to illustrate that iodinated RNA is suitable for obtaining quantitative hybridization data.

Acknowledgments

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Studies on the Combining Region of Protein 460, a Mouse γA Immunoglobulin Which Binds Several Haptens. Binding and Reactivity of Two Types of Photoaffinity Labeling Reagents†

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ABSTRACT: Protein 460, a mouse IgA myeloma immunoglobulin which exhibits binding activity toward the 2,4-dinitrophenyl group, was treated with two photoaffinity labeling reagents. These compounds, 2,4-dinitrophenylalanyl diazoketone and 2,4-dinitrophenyl 1-azide, which do not react with protein 460 in the dark but which have relatively high affinities for the protein, can be photolyzed to give reactive intermediates which have the potential of reacting with amino acid residues that contain heteroatoms. Both 2,4-dinitrophenylalanyl diazoketone and 2,4-dinitrophenyl 1-azide competitively inhibit the binding of ε-2,4-dinitrophenyllysine in the dark. After photolysis, the number of moles of diazoketone reagent incorporated was equivalent to the loss of binding sites for ε-2,4-dinitrophenyllysine. Covalent incorporation of the azide reagent also resulted in loss of binding activity toward 2,4-dinitrophenylalanine. The diazoketone reagent attaches predominantly to the light chain, while the azide reagent reacts chiefly with the heavy chain of protein 460.

An important step in understanding antibody specificity is the elucidation of the tertiary structure of a combining site of an immunoglobulin with defined ligand specificity. To complement the recent high-resolution X-ray crystallographic data on homogeneous immunoglobulins with ligand binding activity (Poljak *et al.*, 1972), efforts to identify and locate some of the amino acid residues comprising the antibody combining regions have been attempted with affinity labeling reagents.

Affinity labeling of antibodies was first tried by Wofsy *et al.* in 1962. They employed reagents based on the hapten used to induce the antibody response, which contained reactive groups capable of forming covalent bonds with side chains of amino acid residues. The structural similarity between the reagent and the hapten permits the reagent to be noncovalently bound in the combining region. It has been assumed that when the reagent is bound in the combining site, it reacts

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