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Two-Stage Photosensitive Label for Antibody Combining Sites*

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ABSTRACT: Dinitrophenylglycine diazoketone, dinitrophenyl alanine diazoketone, and their tritiated analogs were synthesized and used to label, in two steps, the combining sites of rabbit antidinitrophenyl antibodies. Diazoketones are unreactive with proteins, but when photolyzed, they yield carbenes and by rearrangement, ketenes. Such derivatives are reactive with most amino acid side chains. The dinitrophenylamino acid diazoketones enter the antidinitrophenyl site. Excess reagent may then be removed. The diazoketones are then photolyzed within the site, using light of 300–400 mμ. Up to 47% of the sites are covalently labeled. The label is chiefly on the γ-globulin heavy chains, and is limited to the Fab fragments.

The effectiveness of any covalent label for the reactive site of a protein is limited by (i) its ability to localize at the active site and (ii) its covalent reactivity with nearby amino acid residues. One label which has been used for antibodies is the diazonium affinity label (Wofsy *et al.*, 1962, 1967; Metzger *et al.*, 1963a), which has yielded information on those peptides in the combining site containing tyrosine or histidine (Singer *et al.*, 1967; Metzger and Potter, 1968; Koyama *et al.*, 1968).

We have developed another antibody labeling reagent which potentially can react with most amino acid residues (Vaughan and Westheimer, 1969). This reagent, a DNP-diazoketone, enters the anti-DNP combining site. Once in the site, it is converted into reactive carbene and ketene derivatives by ultraviolet irradiation.

Evidence for the synthesis of these labels and their ability to react specifically with the anti-DNP combining site is presented in this paper.

Materials and Methods

Anti-DNP-BGG.[1] New Zealand white male rabbits were immunized in all four footpads with 1 ml of complete Freund's adjuvant containing 5 mg of DNP-BGG (38 moles of DNP/mole of γ-globulin) and 6 mg of desiccated mycobacterium tuberculosis. They were boosted at 5 weeks after immunization with 0.5 mg of alum-precipitated DNP-BGG in PBS in the marginal ear veins. Anti-DNP-BGG was purified from sera of a single animal at 8 and 12 weeks by the method of Farah *et al.* (1960). Fluorescence quenching measurements (Aminco-Bowman or Farrand Mark I spectrofluorometers) gave Q_{max} = 65–75% (uncor) with ε-DNP-lysine (Eisen, 1964). Equilibrium dialysis with [^{3}H]ε-DNP-lysine (Richards *et al.*, 1969) gave an association constant, K_A, = 1.1×10^8 l./mole at 8 weeks. At 12 weeks, $K_A = 1.1 \times 10^8$ l./mole for both [3,5,-6-^{3}H]DNP-D-alanine and [3,5,6-^{3}H]DNP-L-alanine. In the experiments described below, the 8-week anti-DNP-BGG preparation was employed.

DNP-glycine Diazoketone. FDNB (20 ml) in 30 ml of absolute ethanol was added to 10 g of glycine and 42 g of Na_2CO_3 in 250 ml of water and stirred in the dark for 2 hr. The precipitate was washed with ether, redissolved in water, and acidified to pH 1.0 to reprecipitate the DNP-glycine. The infrared spectrum (KBr disk, Perkin-Elmer Model 257 grating infrared spectrophotometer) and melting point after recrystallization from 50% methanol agreed with the literature (Kimmel and Saifer, 1964; Fraenkel-Conrat *et al.*, 1955). A single component was detected on polyamide thin-layer chromatography in solvent systems I and II,[2] paper chromatography (*t*-amyl alcohol saturated with pH 6.0 sodium phthalate buffer), and electrophoresis at pH 1.8, 3.0, and 6.0 (Evered, 1960).

To make the acid chloride, 0.25 g of DNP-glycine was dissolved in 300 ml of dry benzene and 1.0 ml of $SOCl_2$ in 20 ml of benzene was added slowly. The mixture was refluxed for 2 hr and then stored at −20° overnight. The $SOCl_2$ was redistilled (Vogel, 1956) and then could be stored without decomposition in glass-stoppered bottles in a dry box at −20°.

DNP-glycine acid chloride is readily hydrolyzed to DNP-glycine (identified by polyamide thin-layer chromatography in solvents I and II; by electrophoresis at pH 6.0; and by the infared spectrum (Kimmel and Saifer, 1964)). Therefore to determine the yield, the acid choride was used to acylate excess rosaniline acetate, a red amine dye. It was shown that all of the DNP-glycine acid chloride reacts with rosaniline ace-

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[1] Abbreviations used are: BGG, bovine γ-globulin; FDNB, 1-fluoro-2,4-dinitrobenzene; PBS, 0.01 M phosphate buffer (pH 7.4)–0.15 M in respect to NaCl; Q_{max}, maximum quenching of fluorescence.

[2] Solvent I, benzene–glacial acetic acid (80:20, v/v); solvent II, 1-butanol–glacial acetic acid (90:10, v/v).

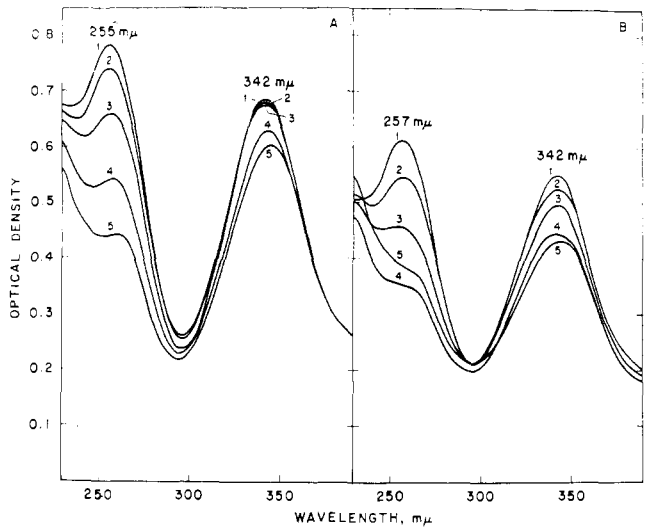

FIGURE 1: Ultraviolet spectra of DNP-L-alanine diazoketone in 1,4-dioxane. (A) Before and during irradiation at 300 mμ (Farrand Mark I spectrofluorometer, xenon arc lamp) (1) 0, (2) 20, (3) 50, (4) 110, and (5) 320 min. (B) Before and during irradiation at 300–400 mμ under six General Electric F15T8-BL lamps (1) 0, (2) 15 (3) 45, (4) 135, and (5) 255 min.

tate, and when the product is subjected to electrophoresis at pH 6.0, there was no yellow spot migrating with DNP-glycine. Thus, when DNP-glycine is synthesized by this method, essentially all of the DNP-glycine is converted into the acid chloride.

DNP-glycine acid chloride also shows a single spot on polyamide thin-layer chromatography in benzene. The infrared spectrum is essentially the same as for DNP-glycine, with the addition of a band at 1780 cm^{-1} attributable to the carbonyl chloride group (Bellamy, 1958).

For synthesis of the diazoketone, 40 mg of the dry acid chloride was dissolved in 100 ml of anhydrous diethyl ether at 0°. Diazomethane (1×10^{-2} mole) in ethereal solution was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich) in a one-piece distillation apparatus (Metalloglass, Boston) behind an explosion-proof shield in a darkened hood (de Boer and Backer, 1963). The acid chloride solution at 0° was added slowly to the cold diazomethane solution, and stored in a stoppered dark container overnight at 4°. DNP-glycine diazoketone precipitated from ether, was collected, and washed with ether at 4°.

DNP-glycine diazoketone was identified by the infrared diazo stretching band at 2115 cm^{-1} (Foffani *et al.*, 1960). The nuclear magnetic resonance spectrum (Varian A-60) in fully deuterated acetone showed all the peaks in the DNP-glycine spectrum, and in addition a broad amino proton triplet at 2.7 ppm below tetramethylsilane and a diazomethyl proton singlet at 6.1 ppm (Dahn *et al.*, 1963). The high-resolution mass spectrum contained an ion peak at 265.0444 mass units ($C_9H_7N_5O_5$, 265.0447) which corresponds to the molecular ion for DNP-glycine diazoketone, and one of equal intensity at 237.0383 ($C_9H_7N_3O_5$, 237.0385) which is consistent with either DNP-glycine carbene or DNP-glycine ketene. DNP-glycine diazoketone is stable at −20°.

Arndt–Eistert Reaction (Bachmann and Struve, 1942). As additional proof of synthesis, DNP-glycine diazoketone was decomposed to the ketene, using heat or silver catalysis, and reacted with methanol to form DNP-β-alanine methyl ester. DNP-glycine diazoketone was heated at 54° in acetone, and then reacted with methanol at −10° for 72 hr. The high-resolution mass spectrum of the product showed a peak at 269.0637 consistent with the molecular ion of DNP-β-AlaMe ($C_{10}H_{11}N_3O_6$, 269.0647). In a separate experiment, DNP-glycine diazoketone in 1 ml of methanol was reacted for 2 hr with 0.05 g of silver benzoate in 1.5 ml of triethylamine (Newman and Beal, 1950). The solution was dried, and 0.5 ml of 50% concentrated HCl–50% glacial acetic acid (v/v) was added. The AgCl precipitate was removed and the supernatant was incubated at 60° for 18 hr. It was dried and taken up in ethanol. Polyamide thin-layer chromatography showed a yellow spot of R_F 0.57 in solvent I and R_F 0.60 in solvent II, which is within 5% of the R_F's measured by us and by Wang and Wang (1966) for DNP-β-alanine.

DNP-L-alanine diazoketone was synthesized by a procedure identical with DNP-glycine diazoketone. The ether solution of the diazoketone was purified by chromatography on basic alumina (Brockman Activity I) and eluted with dioxane. A single component showed on polyamide thin-layer chromatography in solvent I. The infrared spectrum showed a 2115-cm^{-1} diazo stretch band. Low-resolution mass spectrum of the material which volatilizes at 300° showed no peak at *m/e* corresponding to the DNP-L-alanine diazoketone molecular ion. However, the two ions at *m/e* = 210 and 69 are consistent with the two halves, $(DNP\text{-}NHCHCH_3)\cdot^+$ and $(O{=}CCH{=}N{=}N)\cdot^+$. The other ions produced were also consistent with this structure.

[3,5,6-^{3}H]DNP-glycine diazoketone and DNP[β-^{3}H]-DL-alanine diazoketone were synthesized from [3,5,6-^{3}H]FDNB (10.2 Ci/mmole, Amersham Searle) and DL-[β-^{3}H]alanine (40 Ci/mmole, New England Nuclear) as the respective radioactive precursors. A scaled-down version of the above methods was used. Each of the intermediates, and the final products, was identified by thin-layer chromatography with the analogous nonradioactive compounds in solvents I and II. Spots corresponding to the nonradioactive compounds were cut out and their radioactivity was determined.

[3,5,6-^{3}H]DNP-L-alanine and [3,5,6-^{3}H]DNP-D-alanine were synthesized by the procedure for [3,5,6-^{3}H]DNP-glycine. ε-DNP-aminocaproic acid was synthesized according to Carsten and Eisen (1953).

Photolysis Experiments. DNP-L-alanine-diazoketone was dissolved in purified dioxane and the ultraviolet spectrum was recorded on the Cary 15 spectrophotometer. Samples in quartz cells were then exposed to near-ultraviolet irradiation either at 300 mμ in a Farrand Mark I spectrofluorometer or at 300–400 mμ (General Electric Co., 1965) 12 in. below a parallel array of six General Electric F15T8-BL lamps, and the spectra were recorded at regular intervals. Figure 1 shows that with irradiation in the fluorometer the 255-mμ diazoketone absorption band is decreased by 50% in 80 min and stops decreasing after 320 min. Under the G. E. lamps the same band required 30 min to reach a 50% decrease and 90 min to reach a minimum value. The greater spectral changes in the latter case in the 342-mμ DNP band and also at 255 mμ indicate that there may be more than one photochemical reaction taking place.

The products of complete photolysis under these two conditions are being examined in the high-resolution mass spec-

TABLE I: Effect of Irradiation on the Binding of DNP-glycine Diazoketone to Anti-DNP-BGG.

Hapten in Site during Irradiation	Irradiation	[^{3}H]ε-DNP-lysine Incorp (cpm) into Antibody after Irradiation (per nmole of sites)	% of Initial Sites Binding [^{3}H]ε-DNP-lysine after Irradiation
DNP-glycine diazoketone[a]	−	18,082	47
DNP-glycine diazoketone[a]	+	9,638	
ε-DNP-lysine[b]	−	18,715	0
ε-DNP-lysine[b]	+	18,810	

[a] DNP-glycine diazoketone was bound in the anti-DNP site, and the excess hapten was removed. The complex was irradiated at 300–400 mμ for 1 hr. Site-bound hapten was then displaced with 1.2×10^{-5} M [^{3}H]ε-DNP-lysine (15 mCi/mmole). [b] Control experiment binding ε-DNP-lysine to the site, irradiating the complex for 2 hr, then displacing the site-bound ε-DNP-lysine with 1.2×10^{-5} M [^{3}H]ε-DNP-lysine.

trometer (R. E. Lovins, C. A. Converse, and F. F. Richards, 1969, in preparation).

The fluorometer intensity at 300 mμ was determined using a potassium ferrioxalate chemical actinometer (Calvert and Pitts, 1966), and the quantum yield for photochemical decomposition of DNP-alanine diazoketone at 300 mμ was calculated to be 0.22.

Binding Experiments. NONCOVALENT BINDING OF DNP-GLYCINE DIAZOKETONE AND DNP-ALANINE DIAZOKETONE IN THE ANTIGEN COMBINING SITE. Purified anti-DNP-BGG in PBS was incubated 15 min at 37° with twofold molar excess [^{3}H]ε-DNP-lysine, and then layered on a 2 × 25 mm Sephadex G-25 column and eluted with PBS. The elution pattern, showing all the counts in the void volume and little dissociation on the column, is typical of that seen for antibody–hapten complexes of high binding constant (Haber *et al.*, 1965). The same pattern was seen for binding of DNP[β-^{3}H]-DL-alanine diazoketone to anti-DNP-BGG.

A series of equilibrium dialysis cells were set up with increasing concentrations of nonradioactive competing hapten. Figure 2 shows that DNP-glycine diazoketone displaces [^{3}H]ε-DNP-lysine from the [^{3}H]ε-DNP-lysine–anti-DNP-BGG complex almost as well as does ε-DNP-lysine. Thus the DNP-glycine diazoketone competes for the same sites which bind [^{3}H]ε-DNP-lysine, and its binding constant is in the same order of magnitude.

COVALENT BINDING OF DNP-GLYCINE DIAZOKETONE IN THE ANTIGEN COMBINING SITE. Anti-DNP-BGG and DNP-glycine diazoketone were incubated and filtered on Sephadex G-25 as in the above experiments. The peak containing the complex was then put under the ultraviolet lamps for 1 hr; the control was stored in the dark for 1 hr. The complex was incubated overnight with a 12-fold excess of [^{3}H]ε-DNP-lysine, then layered on Sephadex G-25 and eluted to determine the counts incorporated in antibody. In a similar pair of experiments 2-hr ultraviolet irradiation of an ε-DNP-lysine–anti-DNP-BGG complex was followed by incubation with excess [^{3}H]ε-DNP-lysine. Table I shows that photolysis of the DNP-glycine diazoketone in the anti-DNP site causes a 47% decrease in the sites available to [^{3}H]ε-DNP-lysine. Since it was determined that the 12-fold excess of [^{3}H]ε-DNP-lysine is sufficient to displace all noncovalently bound DNP-glycine diazoketone, the 47% loss of binding activity is attributed to covalent attachment of the label in these sites. This cannot be proved until the products of the covalent reaction are identified.

COVALENT BINDING OF DNP[β-^{3}H]-DL-ALANINE DIAZOKETONE IN THE ANTIGEN COMBINING SITE. Anti-DNP-BGG and DNP-[β-^{3}H]-DL-alanine diazoketone were incubated and filtered on Sephadex G-25 as before. The noncovalent antibody–diazoketone complex was eluted. It contained 8.9×10^6 cpm. The complex was irradiated 2 hr, then added to 1×10^{-4} M ε-DNP-lysine, and dialyzed against the same solution overnight. The complex was then dialyzed against five changes of PBS for 2 days, until the concentration of radioactivity outside the sac was 0.2% of that inside. The dialysate contained 1.3×10^6 cpm or 15% of the original noncovalent label.

A portion of the dialysate was mixed with 10 mg of rabbit γ-globulin (Pentex, Inc.), digested with papain (Worthington Biochemical Co.), and chromatographed according to the method of Nisonoff (1964), except the column bed was Whatman CM32 (microgranular CM-cellulose). Figure 3 shows that essentially all the radioactivity is in the Fab peaks,

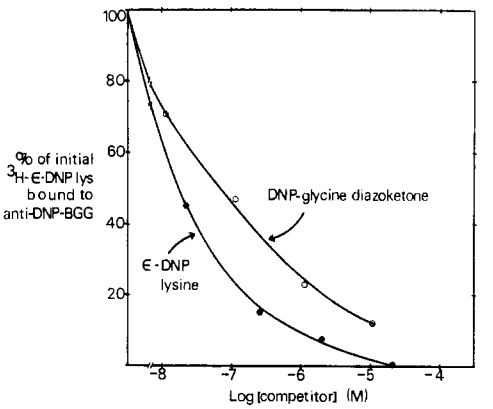

FIGURE 2: Displacement of [^{3}H]ε-DNP-lysine from anti-DNP-BGG by nonradioactive competitors. Anti-DNP-BGG was incubated with [^{3}H]ε-DNP-L-lysine and then chromatographed on Sephadex G-25. Aliquots of the anti-DNP-BGG–[^{3}H]ε-DNP-lysine complex were dialyzed in 1-ml dialysis cells for 18 hr against increasing concentrations of ε-DNP-lysine or DNP-glycine diazoketone. Saturation of the DNP-glycine diazoketone solution was reached at 10^{-5} M.

TABLE II: Distribution of DNP[β-^{3}H]-DL-alanine Diazoketone Label on the Papain Fragments of Anti-DNP-BGG and of Nonspecific Rabbit γ-Globulin.[a]

Protein Labeled	(A) Gel Filtration	Fab I	(B) dpm of Fab II	Fc	(C) Fab I and II (dpm/mg)[c]	(D) Fc (dpm/mg)	(E) Fab I and II/Fc[d]
Anti-DNP-BGG	+	381,000	121,800	5,830	59,200	2,670	22.2
Rabbit γG	+	*b*					
Anti-DNP-BGG	−	443,000	109,500	14,930	103,500	7,830	13.2
Rabbit γG	−	46,000	27,200	8,580	13,500	5,360	2.5

[a] In the first two experiments, the immunoglobulin was incubated with about 1 mole of DNP[β-^{3}H]-DL-alanine diazoketone/mole of protein. The complex was filtered through Sephadex G-25, irradiated 2 hr at 300–400 mμ, and then dialyzed against a 20× molar excess of ε-DNP-lysine. The labeled protein was then mixed with nonspecific γ-globulin, papain digested (Nisonoff, 1964), and chromatographed on Whatman microgranular CM-cellulose. The last two experiments employ the same procedure with the exception of the Sephadex step. [b] Background radioactivity. [c] Assuming optical density = 1.4 for 1 mg/ml of Fab and OD = 1.0 for 1 mg/ml of Fc (Porter, 1959). [d] Specific radioactivity of Fab I and II (column D)/specific radioactivity of Fc (column E).

and that these peaks do not coincide entirely with the OD_{280} peaks (rabbit γ-globulin Fab I and Fab II); 22% of the covalently bound counts was lost by dialysis after papain digestion.

Gel filtration removes all the label from rabbit γ-globulin. In order to show that the high Fab:Fc ratio is not characteristic of all γ-globulin molecules, the gel filtration step was eliminated to increase the binding to nonspecific γ-globulin. The DNP[β-^{3}H]-DL-alanine diazoketone was incubated for 15 min with nonspecific rabbit γ-globulin (Pentex Cohn Fraction II) or with anti-DNP-BGG, photolyzed for 2 hr, and then dialyzed against ε-DNP-lysine and then PBS as before, papain digested, and chromatographed. Table II shows that the high Fab:Fc ratio and high specificity for Fab I is maintained with anti-DNP-BGG even when the gel filtration step is eliminated. However, rabbit γ-globulin shows only about twice as much label in Fab as in Fc. The distribution of the label is over the entire molecule.

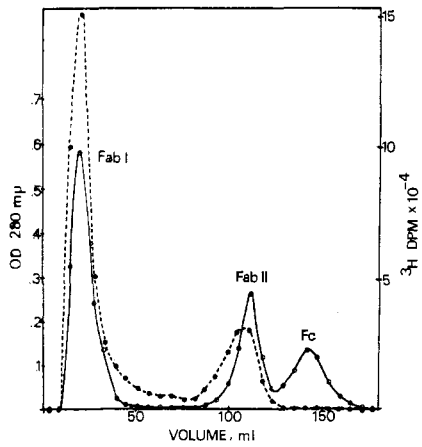

FIGURE 3: CM-cellulose (microgranular, Whatman CM32) separation of Fab I, Fab II, and Fc fragments of DNP[β-^{3}H]-DL-alanine diazoketone labeled anti-DNP-BGG (Nisonoff, 1964). (O—O) Optical density, 280 mμ. The Fc peak is multiplied by 1.4 to correct for its lower absorbance (optical density = 1.4 for 1 mg/ml of Fab and optical density = 1.0 for 1 mg/ml of Fc; Porter, 1959). (●- - -●) ^{3}H dpm × 10^{-4}.

In order to determine the heavy -to light-chain labeling ratio, another portion of the labeled anti-DNP-BGG was mixed with 50 mg of carrier antibody then reduced with 5 mg of dithiothreitol in 3 ml of 0.1 M Tris buffer (0.15 M NaCl–0.002 M EDTA) pH 8.0 for 40 min, and alkylated for 1 hr with 10 mg of iodoacetamide. It was then dialyzed against 1 M propionic acid and chromatographed in 1 M propionic acid on Sephadex G-100. When the counts in the L chain and H + L chain peaks were corrected for yield, the ratio of specific activity in the H and L chains was found to be 4.6:1.

Discussion

When a population of anti-DNP antibodies is reacted with the DNP-diazonium fluoroborate affinity label (Singer *et al.*, 1967), it can be demonstrated that some tyrosine-rich peptides are labeled. We are interested in studying the nature of the combining site of anti-DNP immunoglobulins having restricted heterogeneity (Richards *et al.*, 1969) and of the combining site of mouse myeloma proteins having affinity for the DNP determinant (Eisen *et al.*, 1968; Schubert *et al.*, 1968). It seemed likely to us that we could not depend upon the occurrence of tyrosine or histidine in proximity to the labeling molecule in these immunoglobulins. Therefore we decided to synthesize a reagent which had the potential to label a wider variety of amino acid residues.

To see if the label was located specifically in the combining site, the antibody was digested with papain. Two types of products are produced by this procedure, the Fab and Fc fragments. The Fab fragment comprises the light chain and approximately half of the heavy chain of immunoglobulins; the Fc fragment consists of the other half of the heavy chain. The combining site is known to be in the Fab fragment. The Fab fragments were labeled about 20 times more intensively than the Fc, but when nonspecific rabbit γ-globulin was labeled the ratio was greatly reduced. Thus we interpret the high Fab to Fc labeling ratio for anti-DNP-BGG as being due to the relatively high reactivity of the specific Fab fragment, not to the unreactivity of the nonspecific Fc fragment (Lenard and Singer, 1966).

Since the K_A of our anti-DNP-BGG is heterogeneous, we saturated all the sites by reacting with an excess of diazoketone

hapten, then removing the excess by gel filtration. We found the use of Sephadex G-25 gave the highest Fab to Fc labeling ratios even when less than saturating amounts of reagent were used (Table II), so we have continued to include a gel filtration step in our protocol. However gel filtration is probably not necessary, and should be avoided in cases where the K_A is low.

There are several studies which suggest the type of adducts which diazoketones can form. On photolysis diazoketones tend to form the ketocarbene derivatives. These may react with any amino acid containing a heteroatom (N, O, and S) in the side chain (Kirmse, 1964). Analogous ester carbene labels have been shown to label tyrosine and histidine in this way (Shafer *et al.*, 1966). Some carbenes also insert into C–H bonds (Doering and Knox, 1956) and recently an example of the insertion of an ester carbene into the methyl group of peptide-linked alanine in trypsin has been studied in detail (Vaughan and Westheimer, 1969). If ketocarbenes should also react in this way, the paraffin side chains of amino acid residues may be susceptible to attack.

Wolff (1902) reported the rearrangement of diazoketones to ketenes (Figure 4). Ketene is capable of acylating nucleophilic groups such as carboxyl, hydroxyl, amino, or thiol functions. Neuberger (1938) acylated the side chains of lysine, tyrosine, and cysteine, and suggested that in some cases histidine and aspartic and glutamic acids may also be acylated. Such acylation products would not be expected to survive protein hydrolysis conditions. Preliminary data from hydrolysis of the labeled antibody suggests that at least 50% of the label in the site reacted as the ketene. We do not know what proportion of the products are derived from the carbene. Carbenes have a short life and if the reaction with the protein is slow, substantial portion of the total products may arise from the ketene derivative. Further study of this point is under way.

Both carbenes and ketenes are decomposed by water. Our results show that the combining site of our proteins competes successfully with water for the reagent. There is evidence (Metzger *et al.*, 1963b; Little and Eisen, 1967) that the site complementary to DNP may be hydrophobic, thus reducing the side reaction with water in the region of the site.

The general utility of such labeling reagents has been demonstrated previously by Westheimer's group (Singh *et al.*, 1962; Shafer *et al.*, 1966; Vaughan and Westheimer, 1966) and also by Delpierre and Fruton (1966), Rajagopalan *et al.* (1966), and Knowles and Wybrandt (1968).

A requirement of the label is that it should be sufficiently stable to handle, yet sufficiently photolabile to use in the reaction. DNP-alanine diazoketone was the preferred reagent; it was more stable than the glycine derivative, yet it was photolyzed effectively. The spectrum of DNP-alanine diazoketone shows peaks at 255 and 342 mμ with a shoulder at 400 mμ. Most DNP compounds exhibit an $n \rightarrow \pi^*$ transition between 350 and 360 mμ (molar extinction coefficient ϵ 1.7 $\times$ 10^4, approximately) often with a shoulder around 400 mμ and a weaker band at about 255 mμ (ϵ 8 $\times$ 10^3 approximately) (Fraenkel-Conrat *et al.*, 1955). Diazoketones usually exhibit weak absorption at 350–450 mμ (ϵ 6–100) and one or two stronger bands between 230 and 310 mμ (ϵ 7 $\times$ 10^3–2 $\times$ 10^4) (Kirmse and Horner, 1959). It seems likely that the 255-mμ peak which the DNP-alanine diazoketone shows is the sum of the diazoketo and DNP peaks. On photolysis this peak shifts to the red and is reduced in height to about one-half the original value. This is consistent with our interpretation. High-resolution mass spectroscopy data are also consistent with photolysis of the diazoketone to the carbene or ketene. The quantum yield determined experimentally at 300 mμ (0.22) is in the range of other diazo compounds (Kirmse and Horner, 1959; Mazzucato *et al.*, 1963). Preliminary results indicate that quantum yields at 342 and 400 mμ are smaller.

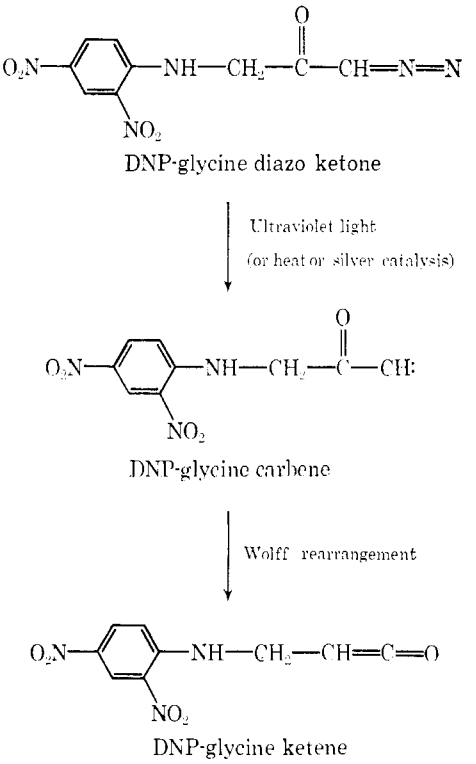

FIGURE 4: Decomposition of DNP-glycine diazoketone to DNP-glycine carbene and subsequent Wolff rearrangement to DNP-glycine ketene. DNP-alanine diazoketone decomposes analogously.

Irradiation of the diazoketone in the anti-DNP site was carried out at 300–400 mμ since antibodies absorb below 300 mμ and may lose hapten binding activity (Tarkchanova *et al.*, *al.*, 1969). No loss of binding activity is seen at 300–400 mμ (Table I).

We have carried out preliminary work which estimates the ratio of labeling of heavy to light chains on heterogeneous anti-DNP rabbit antibodies. Our average figure of 4.6:1 is higher than that 2:1 reported by Singer and Doolittle (1966). Clearly both differences in the antibody population and in the residues labeled would account for the higher ratio.

Our labeling experiments with mouse myeloma proteins which have anti-DNP activity will be reported elsewhere.

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