(1972), *Biochemistry 11*, 4999.
Metzger, H., and Potter, M. (1968), *Science 162*, 1398.
Noelting, E., and Michez, O. (1893), *Ber. 26*, 86.
Poljak, R. J., Amzel, L. M., Avey, H. P., and Becka, L. N. (1972), *Nature (London), New Biol. 235*, 137.
Ray, A., and Cebra, J. J. (1972), *Biochemistry 11*, 3647.
Varga, J., and Richards, F. F. (1973), *Anal. Biochem. 53*, 397.
Vaughan, R. J., and Westheimer, F. H. (1969), *J. Amer. Chem. Soc. 91*, 217.
Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1*, 1031.
Wolff, L. (1902), *Justus Liebigs Ann. Chem. 325*, 129.

Affinity-Labeled Peptides Obtained from the Combining Region of Protein 460. Light Chain Labeling Patterns Using Dinitrophenyl Based Photoaffinity Labels†

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ABSTRACT: Two radioactive photoaffinity reagents, based on the 2,4-dinitrophenyl group, have been used to label a homogeneous mouse myeloma protein (protein 460). After separation of the heavy and light chains, a portion of the light chains was allowed to react with maleic anhydride and then digested with trypsin while another portion was digested directly with trypsin. With one of the reagents, 2,4-[^{3}H]-dinitrophenylalanyl diazoketone, which labels the light chain predominantly, nearly all of the reagent was found attached to an ϵ-amino group of lysine residue 54 *via* an amide linkage. In the case of the other reagent, 2,4-[^{3}H]dinitrophenyl azide, which labels chiefly the heavy chain, only a small fraction (15%) of the reagent which had reacted with the protein was found on the light chain. In contrast to the labeling with the diazoketone reagent, where the reagent attacked a single residue, radioactivity from azide label was found mainly in three light chain peptides which spanned residues 29–58, 62–77, and 78–108.

In a previous paper (Yoshioka *et al.*, 1973), we have reported the labeling of the combining region of protein 460 with two radioactive photoaffinity reagents based on dinitrophenyl (Dnp).[1] This is one of several haptens known to bind to the combining region of this protein (Jaffe *et al.*, 1971; Rosenstein *et al.*, 1972). One Dnp reagent, 2,4-[^{3}H]-dinitrophenylalanyl diazoketone ([^{3}H]Dnp-AD), reacts predominantly with the light chain. The other photoaffinity reagent, 2,4-[^{3}H]dinitrophenyl azide ([^{3}H]Dnp-N_3), is attached predominantly to the heavy chain. This paper deals with the isolation of light chain peptides and the location of the photoaffinity-labeled aminoacid residues in the light chain of protein 460 labeled with [^{3}H]Dnp-AD and [^{3}H]Dnp-N_3.

Materials and Methods

Preparation of Protein 460 and the Separation of Light and Heavy Chains. The purification and the labeling of protein 460 with [^{3}H]Dnp-AD and [^{3}H]Dnp-N_3 have been described in the accompanying paper (Yoshioka *et al.*, 1973). The light and heavy chains were reduced with a 50-fold molar excess of dithiothreitol compared to disulfides in 0.5 M Tris-HCl–6 M guanidine-HCl (pH 8.0) buffer for 4 hr at 50°. Alkylation was effected with a 2.5 molar excess of iodoacetic acid over reducing agent; the pH was maintained by the addition of NaOH. They were then separated on a Sephadex G100 column in 6 M urea–1 M propionic acid. The labeled light and heavy chains were dialyzed exhaustively against 0.2 N acetic acid and lyophilized.

Maleylation Procedure. In a typical experiment 1×10^{-6} mol of labeled light chain was suspended with stirring in 10 ml of 0.2 M phosphate buffer (pH 8.8) and approximately 1×10^{-4} mol of solid maleic anhydride (Butler *et al.*, 1968) was added in five equal portions over a 30-min period. The pH was adjusted with 2.0 N NaOH so that it remained between 8.5 and 9.5. Two milliliters of a 1 N pH 9.0 hydroxylamine solution was added at 20° (Freedman *et al.*, 1968). The mixture was stirred for 2 hr, dialyzed against 0.05 M (pH 8.0) NH_4HCO_3 buffer, and then lyophilized. All the radioactivity was recovered in the maleylated L chain.

Tryptic Digestion of the Dnp-AD-Labeled Maleylated 460 Light Chain. The Dnp-AD-labeled maleylated L chain (0.45 μmol) in 0.2 M NH_4HCO_3 (pH 8.0) buffer was digested with Tos-PheCH_2Cl-treated trypsin (1:200) for 1 hr at 37° and then inactivated with a 2 molar excess of soybean trypsin inhibitor (Schwarz/Mann, Orangeburg, N. Y.). The digest was chromatographed on a 2 × 90 cm Sephadex G50 column in 0.1 M NH_4HCO_3. A peak which accounted for 84% of the total radioactivity was pooled and the material purified by DEAE-cellulose ion exchange chromatography using a 1 ×

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[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; ϵ-Dnp-Lys, ϵ-2,4-dinitrophenyl-L-lysine; Dnp-AD, 2,4-dinitrophenylalanine diazoketone; Dnp-N_3, 2,4-dinitrophenyl 1-azide; Men, menadione (2-methyl-1,4-naphthoquinone); N_2ph-F, 1-fluoro-2,4-dinitrobenzene; *r*, number of moles of reagent bound per 7S monomer of protein 460; Tos-Phe-CH_2Cl, α-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone. BADL, α-*N*-bromoacetyl-ϵ-dinitrophenyl-L-lysine; BADE, *N*-bromoacetyl-*N'*-2,4-dinitrophenyl-L-ethylenediamine.

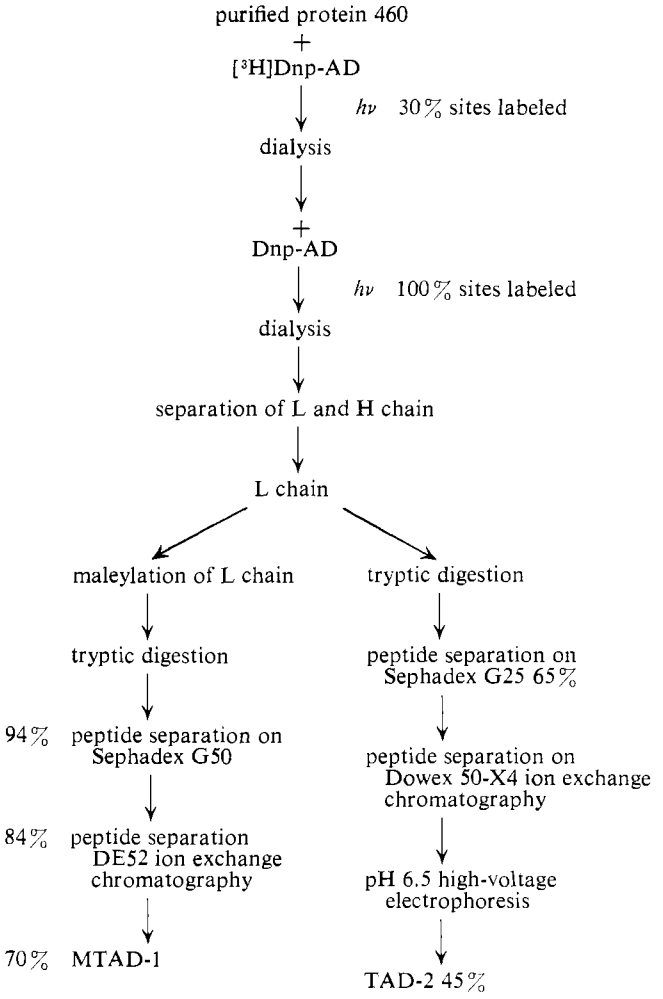

FIGURE 1: Flow sheet of the analytical procedure used to prepare and isolate the Dnp-AD-labeled 460 light chain peptides.

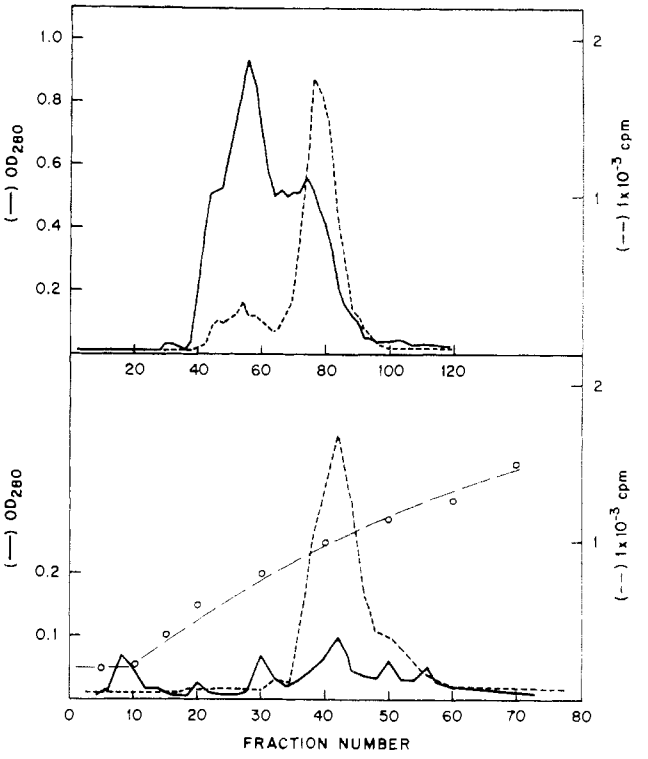

FIGURE 2: (a) Chromatography of maleylated Dnp-labeled protein 460 light chain tryptic peptides on Sephadex G50 column (2 × 90 cm) in 0.1 M NH_4HCO_3. Counting was in Bray's solution without any correction. Fractions indicated above were pooled and lyophilized. (b) DE52 ion exchange chromatography of the pooled radioactive materials in a using a gradient of 0.05 (pH 8.0) to 1.0 M NH_4HCO_3 (pH 8.0).

50 cm DE52 column which was eluted with a gradient from 0.05 to 1.0 M NH_4HCO_3 at pH 8.0 to give the profile shown in Figure 2. A single labeled peptide, MTAD-1, which accounted for approximately 70% of the original radioactivity was isolated.

Tryptic Digestion of the Dnp-AD-Labeled 460 Light Chain. Another batch of Dnp-AD-labeled 460 light chain (1.0 μmol) was digested directly with dicyclohexylcarbodiimide-treated trypsin (Calbiochem) in 0.2 N *N*-ethylmorpholine buffer (pH 8.5) at 37° at a substrate to enzyme ratio of 50:1. After 5 hr, the same amount of trypsin was added and the protein was digested for an additional 5 hr. The digest was then chromatographed on a 2.5 × 137 cm Sephadex G25 column eluted with 2 N acetic acid. The material in the major peak was first chromatographed on a 0.9 × 50 cm Dowex 50 × 4 column with a gradient from 0.1 N pyridine acetate (pH 3.1) to 2.0 N pyridine acetate (pH 5.0) and then purified on pH 6.5 high-voltage electrophoresis using prewashed Schleicher and Schull paper (589 white ribbon).

Amino Acid Analysis. Approximately 2–10 nmol of peptide was hydrolyzed with 300 μl of 5.7 N HCl for 21 hr. The hydrolysate was analyzed on a JEOL 5AH amino acid analyzer equipped with 6-mm cells.

Amino Acid Sequence Determination. The amino acid sequence of the labeled peptide was established by the micro "dansyl-Edman" procedure (Gray, 1967). The dansyl amino acids were analyzed on 5 × 5 cm polyamide thin layer plates (Bruton and Hartley, 1970; Weiner *et al.*, 1972) (Chen-Ching Co., Taiwan).

Results

Isolation of the [³H]Dnp-AD-Labeled Peptides of Proteins 460 Light Chain. Two approaches were used to find the position of the labeled residues (see Figure 1). The larger, maleylated tryptic peptide was used to locate the approximate region of the radioactive residue, while the tryptic peptide served to establish its exact position. Figure 2 gives the separation profile used for the isolation of the maleylated tryptic peptide MTAD-1. The labeled peptide MTAD-1 accounted for approximately 70% of the total radioactivity in the L chain. This peptide had amino-terminal serine and carboxy-terminal arginine. The amino acid composition of MTAD-1 is shown in Table I. Peptide MTAD-1 has 34 amino acids with the partial amino acid sequence shown in Figure 3. It spans residues 25–58 of the light chain and was placed by homology with the known regions of protein 460 (Haimovich *et al.*, 1972). This peptide includes both the first and the second "hypervariable" regions of the light chain (Wu and Kabat, 1970). An independent tryptic digest of the labeled light chain without maleylation was also performed. High-voltage electrophoresis of the whole tryptic digest at pH 6.5 (Figure 4) revealed a basic peptide which accounted for 65% of the radioactivity in the digest. This basic peptide TAD-2 was purified first on Sephadex G25 (Figure 4) followed by Dowex 50-X4 ion exchange chromatography and finally by pH 6.5 high-voltage electrophoresis. Peptide TAD-2 has the tentative sequence Lys-Val-Ser-Asn-Arg, which is also the C-terminal portion of peptide MTAD-1 (see also Figure 3).

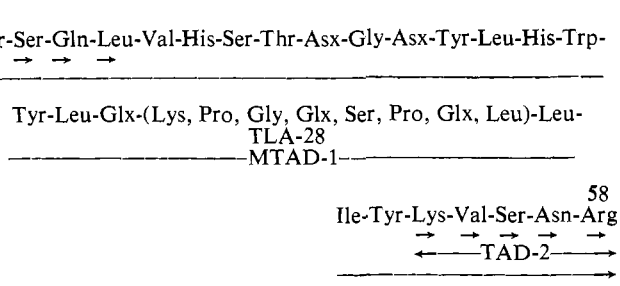

FIGURE 3: The tentative partial amino acid sequence of protein 460 light chain around residues 25–58 (Haimovich *et al.*, 1972). We have placed peptides MTAD-1, TAD-2, and TLA-28 according to this sequence.

Location of the Dnp-AD-Labeled Residue. When peptide TAD-2 (Lys-Val-Ser-Asn-Arg) was subjected to sequential Edman degradation, 98% of the total radioactivity was lost after the first reaction cycle. This could mean that the radioactivity was on the N-terminal lysine residue (54) and was lost due to extraction of lysine phenylthiazolinone. Alternatively, the bond produced by the radioactive carbene, or ketene, with protein 460 could be unstable under the conditions of the Edman degradation and could be lost on extraction, in a form not associated with an amino acid residue. To distinguish between these possibilities, the maleylated peptide MTAD-1 which comprises residues 25–58 and contains the label, was subjected to a single cycle of Edman degradation. Only 3% of the total radioactivity was extracted with the NH_2-terminal serine phenylthiazolinone, showing that the label remained attached to the peptide. Thus, it was concluded that Lys-54 in peptide TAD-2 was the residue labeled by the [^{3}H]Dnp-AD reagent.

At pH 6.5, the mobility (μ) of the labeled pentapeptide TAD-2 (Lys-Val-Ser-Asn-Arg) was equal to 0.3 and at pH 1.9 it was equal to 0.88 (Offord, 1966). These mobilities indicate the presence of a single net positive charge at pH 6.5 and of two positive charges at pH 1.9. A molecular weight of 840 was assumed for the peptide. If the ϵ-amino group of the lysine were functional either as a primary or secondary amine, an additional positive charge would have been expected both at pH 6.5 and 1.9.

The absence of these additional positive charges indicates that the link between [^{3}H]Dnp-AD and the lysine is *via* the ϵ-amino group, and that this is not an insertion reaction (see Yoshioka *et al.*, 1973) which would be expected to produce a secondary amine, but rather an acylation of the ϵ-amino group by the ketene intermediate formed by the Wolff rearrangement of [^{3}H]Dnp-AD (see Figure 5). The linkage of [^{3}H]Dnp-AD to lysine is unstable in 6 N HCl at 110° for 24 hr, since a single lysine residue is regenerated by such treatment (Table I). The charge and stability characteristics strongly support the presence of an amide linkage.

Labeled Peptides Derived from the Reaction of [^{3}H]Dnp-N_3 with the Protein 460 L Chain. Between 13 and 15% of the radioactivity incorporated in [^{3}H]Dnp-N_3 labeled protein 460 was located in the L chain. Radioactive L chain was fractionated using the same techniques as indicated in Figures 1 and 2 for the maleylated [^{3}H]Dnp-AD labeled L chain. Two-dimensional peptide maps using high-voltage electrophoresis at pH 6.5 in one direction and chromatography in the second direction were performed on the tryptic digest. Three labeled peptides were isolated. Table II shows the partial sequence of

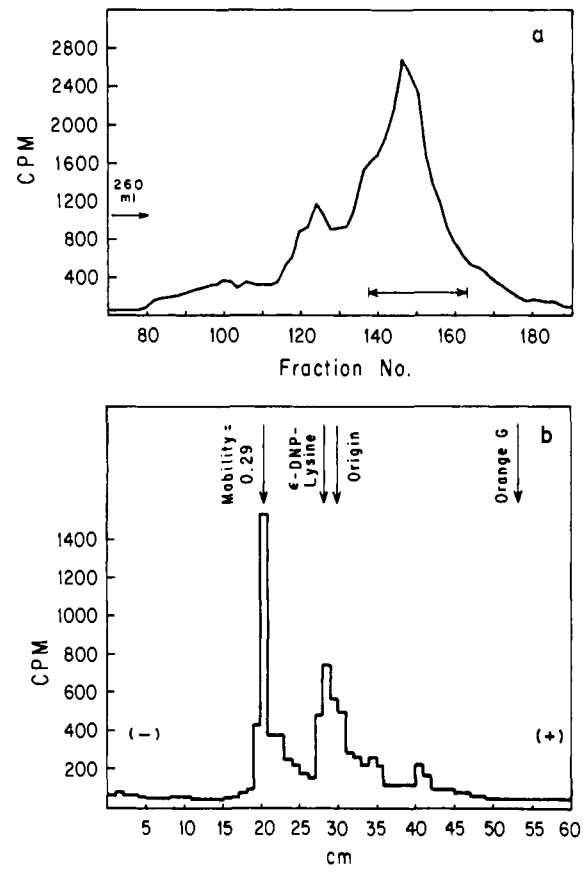

FIGURE 4: (a) Chromatography of the Dnp-labeled protein 460 light chain tryptic digest on a Sephadex G25 fine column 2.5 × 137 cm in 2.0 N acetic acid. The fractions indicated were pooled and later purified on Dowex 50-X4 ion exchange chromatography and high-voltage electrophoresis at pH 6.5. (b) High-voltage electrophoresis of the Dnp-labeled protein 460 light chain tryptic digest at pH 6.5 and 3 kV for 2 hr. A 1-cm strip was cut and counted in 5 ml of toluene–Liquifluor (New England Nuclear) scintillation fluid without any correction.

TABLE I: Amino Acid Composition of Dnp-AD-Labeled Maleylated Tryptic Peptide MTAD-1 and Tryptic Peptide TAD-2.

	MTAD-1	TAD-2
Asp	4 (3)	1
Thr	3 (1)	
Ser	5	1
Glu	4	
Pro	2	
Gly	2	
Ala	1 (0)	
Val	2	1
Ile	1	
Leu	5	
Tyr	2 (3)	
Phe	0	
Trp	Not determined	
Lys	2	1
His	2	
Arg	1	1
Total residue	34	5
N terminal	Ser	Lys
C terminal	Arg	Arg

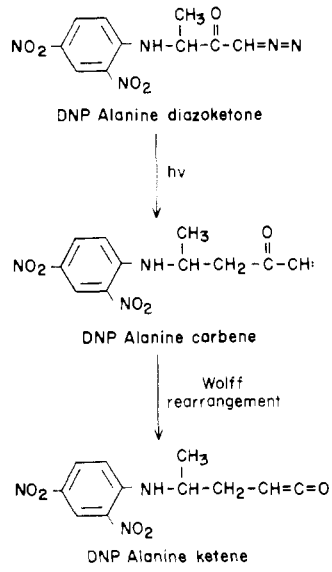

FIGURE 5: Photolysis of Dnp-AD to dinitrophenylanalylcarbene and subsequent rearrangement to dinitrophenylalanylketene.

these peptides and their probable positions in the L chain. Peptide TLA-28 was clearly identified as containing residues 25–58 and, hence, is identical in sequence with peptide MTAD-1. Not enough of this labeled peptide was available to unequivocally identify the labeled residue, but a tryptic "fingerprint" map of the [^{3}H]Dnp-N$_3$-labeled protein 460 L chain (nonmaleylated) gave a radioactive peptide identical in mobility with the radioactive pentapeptide TAD-2 (residues 54–58) obtained from the [^{3}H]Dnp-AD experiment. It is possible, therefore, that the same Lys-54 residue is labeled by the nitrene reagent as by the diazoketone reagent, although clearly the efficiency of labeling is very low. In addition, two other peptides were labeled: peptide TLA-31 comprising residue 78–108 and peptide TLA-40, which has not yet been accurately placed, but is probably either 55–61 or 62–77 and has the partial sequence Phe-Ser-Gly(Val,Glx,Thr)-?-Arg. Peptides TLA-31 and TLA-40 were placed by comparison with the sequence of protein 460 light chain as determined by L. Hood and collaborators (personal communication). All three labeled peptides were also available only in minute quantities, making identification of the labeled residues impossible. It is, however, clear that the nitrene reagent labels residues in several peptides of the variable region in the L chain.

Discussion

Protein 460 binds both the dinitrophenyl group and menadione with substantial energies of interaction (Eisen, 1971). An ideal affinity reagent for labeling a combining site in a protein should have the following characteristics. (a) It should label the protein only when that portion of the reagent having affinity for the protein is rigidly fixed in the combining site. (b) The group which reacts to form a covalent bond with the protein should be capable of reacting with as many amino acid side chains or components of the peptide backbone as possible. If reactivity is confined to only a few residues, there is a possibility that highly reactive residues in the combining region, not necessarily in or near the specific site, will become labeled upon release of the hapten from the combining site. (c) The group reacting with the protein should be as close as possible to the portion of the reagent having affinity for the protein. Dnp affinity reagents have been used for myeloma proteins in which the distance from the center of the dinitrobenzene ring to the end of the reactive group in the fully extended conformation varies from 3.4 Å (the active dinitrophenylnitrene) to 15.3 Å for the bromoacetyl reagent BADL (Weinstein *et al.*, 1969). A sphere swept out by a radius of 15.3 Å would encompass a substantial portion of the Fab fragment of NEW, an immunoglobulin whose dimensions are accurately known (Poljak *et al.*, 1972). Table III gives the dimensions of some of the affinity reagents which have been employed to label the combining sites of myeloma proteins and antibodies with anti-Dnp activity. The Dnp-N$_3$ affinity labeling reagent exhibits favorable characteristics with respect to reactivity and the distance between the reactive and binding moieties. However, because of the closeness of the azide group to the Dnp ring, the reagent may not be able to assume the proper orientation for reaction or to reach an amino acid residue side chain while rigidly fixed in the combining site. The small amount of light chain labeling which occurs is found in three portions of the variable region. This labeling pattern is probably not consistent with that expected from a rigidly held affinity labeling reagent and suggests that these residues have become labeled when the reagent dissociates from the combining site. The diazoketone reagent, as the reactive ketene intermediate, has in the extended form a distance of 6.5 Å from the center of the dinitrobenzene ring to the reactive ketene group. This reagent reacts in high yield with Lys-54, the same residue that was labeled by Haimovich *et al.* (1972) in protein 460 using BADE, a reagent measuring 9.5 Å from the center of the dinitrobenzene ring to the active methylene group.

Irradiation of aliphatic diazoketones produces first a carbene which is known to insert into C–O, C–N, S–H, N–H, or C–H linkages and such insertions have been demonstrated to occur in proteins although with relatively low yield (Vaughan and Westheimer, 1969). A Wolff rearrangement may also occur which results in the shift of the oxygen onto the terminal carbon of the chain, producing a ketene capable of acylating nucleophiles such as carboxyl, hydroxyl, amino, or thiol groups. It is interesting to note that the primary photolysis product of Dnp-AD does not react with the light chain and that nearly all of the labeling is due to the acylation reaction

TABLE II: 460 Light Chain Peptides Labeled by [^{3}H]Dnp-N$_3$.

Peptide	Partial Sequence	% of Total Radioact
TLA-28	Ser-Ser-Gln-Leu···Arg 25 58	4
TLA-31	Val-Glx-Ala···Arg 78 108	5
TLA-40	Phe-Ser-Gly·····Arg	3

TABLE III: Dimensions of Some Mono- and Dinitrophenyl Affinity Labeling Reagents.

Reagent	Dimension (Å)	Position
Dnp-AD	6.5	460 L, Lys-54
Dnp-N$_3$	3.14	460 L, Lys-54
BADE	9.15	460 L, Lys-54
		315 L, Tyr-34
BADL	12.94	315 H, Lys-54

rather than the insertion reaction. One possible explanation is that the rate of reaction of the carbene with the protein is slow compared with the rate of rearrangement so that the acylation reaction dominates. An alternate possibility is that the reagent is unable to assume the proper orientation for reaction, or there may be no suitable heteroatomic residues within reach of the carbene intermediate, while the Dnp-AD is rigidly held in the combining site. Dissociation of the reagent from the site followed by rearrangement to the ketene would then precede incorporation. Once the reagent has dissociated from the combining site, its reaction with an amino acid side chain may be indicative of the proximity of the residue to the combining region or result from the residue being more nucleophilic than the surrounding residues. Thus, it is very difficult to distinguish between the situation where a label is held *in situ* and a residue is labeled due to the proximity of the residue to the reactive group of the reagent, and the situation where the reagent dissociates and combines by covalent linkage to a highly reactive residue near, but not at, the combining site.

Despite the fact that Dnp-AD has a different reactive group from the reagent used by Haimovich *et al.* (1972), it nevertheless reacts with the same residue in the light chain of protein 460. The evidence that the reaction of [^{3}H]Dnp-AD with protein 460 is site directed (Yoshioka *et al.*, 1973) suggests that Lys-54 is not an abnormally reactive lysine in the protein, but must be located at or close to the combining site.

A subsequent paper will report our studies on the protein 460 heavy chain labeled with Dnp-N_3.

Acknowledgments

We would like to thank Dr. Martine Y. K. Armstrong and Miss Louise Camera for the production of sera containing protein 460. We are also grateful to Mrs. Pat Cellini for her expert technical assistance.

References

Bruton, C. J., and Hartley, B. S. (1970), *J. Mol. Biol. 52*, 165.

Butler, P. J. G., Harris, J. H., Hartley, B. S., and Leberman, R. (1969), *Biochem. J. 112*, 679.

Eisen, H. N. (1971), *Progr. Immunol.*, 243.

Freedman, M. H., Grossberg, A. L., and Pressman, D. (1968), *Biochemistry 7*, 1941.

Gray, W. R. (1967), *Methods Enzymol. 11*, 139.

Haimovich, J., Eisen, H. N., Hurwitz, E., and Givol, D. (1972), *Biochemistry 11*, 2389.

Jaffe, B. M., Simms, E. S., and Eisen, H. N. (1971), *Biochemistry 10*, 1693.

Offord, R. E. (1966), *Nature (London), 211*, 59.

Poljak, R. J., Amzel, L. M., Avey, H. P., and Nisonoff, A. (1972), *Nature (London), New Biol. 235*, 137.

Rosenstein, R. W., Musson, R. A., Armstrong, M., Konigsberg, W. H., and Richards, F. F. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 877.

Vaughan, R. J., and Westheimer, F. H. (1969), *J. Amer. Chem. Soc. 91*, 217.

Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.

Weinstein, Y., Wilchek, M., and Givol, D. (1969), *Biochem. Biophys. Res. Commun. 35*, 694.

Wu, T. T., and Kabat, E. A. (1970), *J. Exp. Med. 132*, 211.

Yoshioka, M., Lifter, J., Hew, C-L., Converse, C. A., Armstrong, M. Y. K., Konigsberg, W., and Richards, F. F. (1973), *Biochemistry 12*, 4679.