Affinity Labelling of the Binding Site of Rabbit Antibody

EVIDENCE FOR THE INVOLVEMENT OF THE HYPERVARIABLE REGIONS OF THE HEAVY CHAIN

By CHRISTOPHER E. FISHER and ELIZABETH M. PRESS

*Medical Research Council Immunochemistry Unit, Department of Biochemistry,
University of Oxford, Oxford OX1 3QU, U.K.*

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The binding sites of rabbit antibodies with affinity for the haptenic group 4-azido-2-nitro-phenyl-lysine have been specifically labelled by photolysis of the hapten-antibody complex. The extent of covalent labelling was 0.5–0.9 mol of hapten bound/mol of antibody and, by using an immunoadsorbent, antibody with 1.3 mol of hapten/mol was obtained. The antibody was specifically labelled in the binding site and the ratio of labelling of heavy and light chains was in the range 3.3–5.0. The labelled heavy chains were cleaved by CNBr treatment and after reduction and alkylation of the intrachain bonds, were digested with trypsin. Evidence is presented that two regions of the heavy chain, positions 29–34 and 95–114, together contain about 80% of the label on the heavy chain; these two regions respectively include two of the hypervariable regions of rabbit heavy chain.

It is known that the Fab fragment* of an antibody contains the antigen-binding site. The assumption that the binding site was within the variable region of the fragment received formal proof when the Fv fragment of a Dnp-lysine-binding mouse myeloma protein (MOPC 315) was shown to retain full affinity for the ligand (Inbar *et al.*, 1972). Amino acid sequence studies on both myeloma proteins and on normal immunoglobulins have revealed the existence of hypervariable regions within the variable regions of heavy and light chains (see Kabat & Wu, 1971). It is generally agreed that the structural basis of antibody specificity resides in primary structure, and involvement of the hypervariable regions in the binding site is a reasonable proposition.

In the method of affinity labelling of antibodies, a hapten, with a chemically reactive group, is covalently bound in the binding site and in this way direct evidence can be obtained of the sections of the peptide chains contributing to the site. The diazonium group has been extensively used as the chemically reactive group, incorporated into haptens such as benzene arsonate and nitrobenzene. Both induced antibodies and myeloma proteins with affinity for a group such as Dnp-lysine have been labelled, and in many

instances tyrosine has been identified as the labelled residue. In some cases the position of the residue in the sequence has been established (e.g. Thorpe & Singer, 1969; Ray & Cebra, 1972; Goetzl & Metzger, 1970). The criticisms that can be raised against this reagent, however, are that it is not a part of the haptenic group and that only a very limited number of amino acids are susceptible to attack by the diazonium group. Another derivative used in affinity labelling is bromoacetyl; a series of reagents were made in which this group was placed at different distances from the Dnp group and these reagents were used to 'map' the binding site of both induced antibodies and the myeloma protein MOPC 315 (Strausbauch *et al.*, 1971). However, although this approach has been successful in defining parts of the peptide chains involved, it is still open to the criticisms that only a limited number of amino acids are susceptible to attack, and that labelling may occur peripheral to the site. In contrast with these selective labelling reagents, the nitrenes are, in principle, able to insert into any C–H bond and aryl nitrenes can be generated photochemically from their stable precursors, aryl azides. Fleet *et al.* (1969, 1972) described the hapten ϵ -(4-azido-2-nitrophenyl)-L-lysine (Nap-lysine) and the affinity labelling of specific antibody by photolysis of the antibody-hapten complex. The merits of this reagent are that the chemically reactive group is a part of the specific determinant and that it is non-selective in its attack on amino acids so that any labelling should occur in the binding site and not peripheral to it. In the present paper we report on the labelling of anti-Nap antibody and anti-'Dnp' anti-

* Abbreviations: Fab fragment, *N*-terminal half of heavy chain and light chain; Fv fragment, variable regions of heavy and light chains; Fc fragment, *C*-terminal half of heavy-chain dimer; Nap, 4-azido-2-nitrophenyl; $\square$ Glu, CmCys, Hse and ITyr (in amino acid sequences and tables), pyrrolid-2-one-5-carboxylic acid, *S*-carboxymethylcysteine, homoserine and moniodotyrosine; IgG, immunoglobulin G.

body; the latter was a fraction of anti-Dnp antibody which was precipitable with Nap-human serum albumin. We present evidence for the involvement of two hypervariable regions of the heavy chain in the binding site for ϵ -Nap-lysine. A report on some of these results was given in Press *et al.* (1971).

Experimental

Materials

The reagents used were as described in Fleet *et al.* (1972). Dinitrophenol was recrystallized from ethanol. ϵ -Dnp-Lysine (chromatographically pure reagent) and citraconic anhydride were both supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. Trypsin, treated with chloromethyl L-(1-tosylamido-2-phenyl)ethyl ketone, chymotrypsin and carboxypeptidases A and B were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pepsin was a gift from Dr. A. P. Ryle, Edinburgh, U.K. Aminopeptidase M (Röhm G.m.b.H.) was obtained from Hexoran Co. Ltd., Unity Works, Derwent St., Belper, Derby., U.K. The reagents for the manual sequential Edman degradation were all redistilled and stored at -20°C .

Nap-bovine γ -globulin. This was prepared by a similar method to that described by Fleet *et al.* (1972), except that the amount of 4-fluoro-3-nitrophenyl azide was increased. Three additions of 100mg of 4-fluoro-3-nitrophenyl azide in 1 ml of acetone were made over a period of 40h, to a solution of 100mg of bovine γ -globulin in 8 ml of 0.1M-sodium borate buffer, pH9.8, containing 3mM-EDTA. The reaction mixture was protected from light and stirred at $40-45^{\circ}\text{C}$. The product was centrifuged and the supernatant dialysed against 0.1M-Tris-HCl buffer, pH8.0, containing 3mM-EDTA. The dialysed protein was stored frozen. The number of mol of Nap groups/mol of protein, determined by amino acid analysis, was 40-45. Lysine was the only residue to be substituted.

Nap-human serum albumin. This was prepared by the reaction of 250mg of human serum albumin in 20 ml of 0.1M-sodium bicarbonate-carbonate buffer, pH9.0, containing 2mM-EDTA with 750mg of 4-fluoro-3-nitrophenyl azide in 15 ml of acetone, added in two portions over a period of 24h. The reaction mixture was protected from light and stirred at 50°C . The product was centrifuged and the supernatant dialysed against 0.1M-ammonium bicarbonate buffer, pH8.5, containing 2mM-EDTA. The dialysate was freeze-dried and analysed. The number of mol of Nap groups/mol of protein was about 30 and only the lysine residues were substituted.

Dnp-bovine γ -globulin and Dnp-human serum albumin. These were both prepared by the method of Little & Eisen (1967). Amino acid analysis showed the presence of 60mol of Dnp groups/mol of Dnp-bovine

γ -globulin and 52mol of Dnp groups/mol of Dnp-human serum albumin, on the lysine residues.

Dnp-lysyl Sepharose 4B. This was made by the general method of Cuatrecasas *et al.* (1968). Sepharose 4B [180ml; Pharmacia (GB) Ltd., London W.5, U.K.] was diluted to 300 ml with water and the pH adjusted to 11.0 by the addition of 4M-NaOH. Crushed CNBr (750mg) was added with stirring and the pH maintained at 11.0 for 10min. The activated Sepharose was washed rapidly with 3.5 litres of ice-cold 0.1M-sodium bicarbonate buffer, pH9.0, suspended in 180ml of the same buffer and 60mg of ϵ -Dnp-lysine in 30 ml of buffer was added. The mixture was stirred at 4°C overnight and then washed successively with 0.1M-Tris-HCl-0.14M-NaCl buffer, pH7.4, until the eluate was colourless, and then with 0.1M-acetic acid-0.14M-NaCl and 0.1M-Tris-HCl-0.14M-NaCl buffer, pH7.4. The immunoadsorbent was packed in a column (diam. 5.5cm), stored in the cold room at 4°C and washed with the same sequence of buffers before and after use.

Methods

Immunization. Rabbits homozygous at the *a* locus for the Aa3 allotype were immunized with either Nap-bovine γ -globulin or Dnp-bovine γ -globulin as described by Fleet *et al.* (1972). The anti-Nap antibody response was 1-1.5mg of antibody precipitable with Nap-human serum albumin/ml of serum. The anti-Dnp antibody response was 0.6-1.4mg of antibody precipitable by Dnp-human serum albumin/ml, of which 70-80% was precipitable by Nap-human serum albumin.

Isolation of anti-Nap antibody. Antibody to bovine γ -globulin could not be detected by the technique of double diffusion in agar (Ouchterlony, 1953) but anti-Nap antisera were adsorbed as a routine with 5mg of bovine γ -globulin/100ml of antiserum. Pooled adsorbed antisera containing up to 400mg of anti-Nap antibody, as measured by the precipitin reaction with Nap-human serum albumin, were applied to a Dnp-lysyl-Sepharose column (diam. 5.5cm; vol. 150ml; equilibrated with 0.1M-Tris-HCl-0.14M-NaCl buffer, pH7.4, at room temperature; flow rate 200ml/h). The serum that was eluted with the buffer contained no anti-Nap antibody and the column was washed with buffer until the extinction at 280nm had fallen to that of the eluting buffer. Usually about 1 litre of buffer was needed. The adsorbed antibody was eluted with 400ml of 0.1M-dinitrophenol in the buffer. About 75-80% of the anti-Nap antibody was recovered in the eluate and the remainder could be eluted with 100ml of a solution of 1mM- ϵ -Dnp-lysine in 0.1M-acetic acid-0.14M-NaCl. The yellow solutions containing the antibody were passed down a column of Sephadex G-25 in 0.14M-NaCl to remove the Dnp reagents. The antibody solutions were concentrated

by ultrafiltration in an Amicon ultrafiltration cell to 10mg/ml, dialysed against 0.1M-Tris-HCl-0.14M-NaCl and stored frozen. The affinity constant of the antibody for ϵ -Nap-[3 H]lysine, as measured by equilibrium dialysis (Eisen, 1964), was in the range 5×10^6 – 1×10^7 M $^{-1}$ at 4°C.

Isolation of anti-'Dnp' antibody. The method of Eisen *et al.* (1967) was used. Anti-Dnp antisera were precipitated at equivalence with Nap-human serum albumin and the resulting precipitate redissolved by stirring at 20°C for 12 h with a saturated solution of 4-azido-2-nitrophenol in 0.03M-sodium phosphate buffer, pH7.3, containing 10% (v/v) dimethylformamide. A 1ml portion of solution was sufficient to redissolve 4 mg of precipitated antibody. The solution was centrifuged and the remaining precipitate again stirred at 20°C for 3 h with one-fifth of the original volume of 4-azido-2-nitrophenol. The combined supernatants were applied to a layered column of DEAE-cellulose packed on top of Dowex 1 (X8) equilibrated with 30mM-sodium phosphate buffer, pH7.3, and the pure anti-'Dnp' antibody was eluted from the column in a yield of 40–60% of the antibody precipitable by Nap-human serum albumin present in the serum. The antibody was concentrated to 5mg/ml and dialysed against 0.03M-sodium phosphate buffer, pH7.3, and stored frozen. The affinity constant of the anti-'Dnp' antibody for ϵ -Nap-[3 H]lysine was 5×10^5 M $^{-1}$ at 4°C.

The methods for separation of the chains, CNBr treatment of heavy chains, fractionation of fragments and total reduction and alkylation with iodo[14 C]-acetic acid were all as described by Fleet *et al.* (1972). Citraconylation, tryptic digestion, removal of the blocking groups from the lysine residues and fractionation of the tryptic peptides were carried out as described by Mole *et al.* (1971). High-voltage electrophoresis at pH6.5, 3.5 and 1.9 was performed as described by Cebra *et al.* (1968). Radioautography of the *S*-carboxymethylcysteine-containing peptides was performed as described by O'Donnell *et al.* (1970). Scintillation counting was done with a Nuclear Enterprises model NE8312 liquid-scintillation counter and scintillation fluid NE250 (Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.).

Results

Photolabelling of antibody

Anti-Nap antibody and anti-'Dnp' antibody were both affinity labelled with the radioactive hapten (ϵ -Nap-[3 H]lysine) of specific radioactivity between 1.5×10^7 and 2.5×10^7 c.p.m./ μ mol. To ensure that photolabelling of antibody was specific, i.e. the hapten was covalently bound only in the antigen-binding site, the following procedure was adopted as a routine. Antibody (4–10mg/ml) in either 0.03M-sodium phos-

phate buffer or 0.1M-Tris-HCl-0.1M-NaCl, pH7.4, containing 3mM-EDTA was incubated with four times the molar amount of radioactive hapten in the dark at 37°C for 1 h, and then at 2°C overnight. The mixture was then chromatographed on a column of Sephadex G-25 in the appropriate buffer containing 0.1 μ M-hapten at 2°C in the dark. The antibody was eluted at the void volume, with 2.0mol of hapten bound/mol, as calculated from E_{280} and the radioactivity. Excess of hapten was eluted at about four times the void volume. The antibody-hapten complex was photolysed at 2°C overnight by two Mazda 125W MB/V pearl-glass lamps immersed in a solution of NaNO₂ (0.5%, w/v) to absorb radiation of wavelengths shorter than 400nm. The photolysed antibody complex was then dialysed for 3 days against 0.5M-NaCl to remove non-covalently bound hapten. Dialysis failed to remove all the non-covalently bound hapten, but the amount covalently bound could be determined by precipitating 0.5mg of antibody with 2vol. of 10% (w/v) trichloroacetic acid containing 20% (v/v) acetone at 2°C, washing twice with the trichloroacetic acid-acetone solution at 2°C, redissolving the antibody in 0.1M-NaOH and counting the solution for radioactivity. The extent of labelling varied with different pools of antibody, 0.5–0.8mol of label/mol of anti-Nap antibody and 0.8–0.9mol of label/mol of anti-'Dnp' antibody. This was considerably lower than previously reported (1.3mol of label/mol of antibody; Fleet *et al.*, 1972), but when the partially labelled antibody was reincubated with ϵ -Nap-[3 H]lysine, although non-covalent binding brought the total bound hapten to 2.0mol/mol, on photolysis only a 10–20% increase in labelling could be achieved.

To investigate this further, partially labelled anti-Nap antibody was fractionated into a highly labelled and a poorly labelled fraction by recycling through the Dnp-lysyl-Sepharose immunoabsorbent column used in the isolation of anti-Nap antibody. Now, 30% of the antibody and 60% of the radioactivity were not retained on the immunoabsorbent column. This fraction, *x*, was labelled to the extent of 1.3mol/mol of antibody. The other 70% of the antibody, fraction *y*, containing 40% of the radioactivity was eluted with 0.1M-dinitrophenol and was labelled to the extent of 0.33mol of hapten/mol of antibody. To establish whether or not the label was bound in the specific binding site in this latter fraction, it was digested with papain giving rise to the two univalent Fab fragments (Porter, 1959) and the digest recycled through the immunoabsorbent column. Although 85% of the Fab fragments were retained on the column, only 15% of the radioactivity was retained (see Scheme 1). Presumably fraction *y* contains non-symmetrically labelled antibody molecules but these can be symmetrically labelled on re-incubation with hapten. More than 90% of the label therefore is

Scheme 1. Fractionation of partially labelled antibody by recycling through a Dnp-lysyl-Sepharose immunoabsorbent column

Fraction x was not adsorbed to the column. The column was eluted with 0.1 M-Tris-HCl-0.14 M-NaCl buffer, pH 7.4, and when the extinction at 280 nm had fallen to that of the eluting buffer, the adsorbed fraction y was eluted with 0.1 M-dinitrophenol in the same buffer. A portion of fraction y was digested with papain (Porter, 1959) and the digest recycled on the immunoabsorbent column. Another portion was relabelled.

Table 1. Distribution of label on the heavy and light chains isolated from three preparations of affinity-labelled antibody

Antibody preparation	Antibody (μ mol)	For details see the text.		
		Ratio of mol of label/mol of antibody	Ratio of mol of label/mol of heavy chain	Ratio of mol of label/mol of light chain
1a	7.8	0.65	0.28	0.07
1b	4.0	1.30	0.50	0.11
2	30.0	0.80	0.33	0.10

covalently bound in the antigen-binding site and this high specificity of labelling was confirmed by calculating the valency of the partially labelled antibody, from equilibrium-dialysis studies on the labelled antibody.

Since highly labelled antibody had some advantages for the isolation of labelled peptides, one large batch of labelled anti-Nap antibody (18 μ mol) was fractionated on the immunoabsorbent column. Fraction x

(yield 4 μ mol) with 1.3 mol of label/mol of antibody is one of the affinity-labelled antibody preparations (1b) used to locate the antigen-binding site.

The results with three different pools of affinity-labelled antibody will be described: pool 1a, anti-Nap antibody with 0.65 mol of label/mol of antibody; pool 1b, anti-Nap antibody with 1.3 mol of label/mol of antibody; pool 2, anti-'Dnp' antibody with 0.8 mol of label/mol of antibody (see Table 1).

Fig. 1. (a) Rabbit heavy chain and (b) rabbit heavy-chain fragment C-1, positions 1-251

(a) The positions of methionine residues are shown by full bars and fractional methionine residues by half bars. CNBr cleavage gives rise to the fragments C-1, C-3, C-4, C-5 and C-6. The intrachain disulphide bridges shown by full lines were intact in the heavy chain isolated from partially reduced and alkylated antibody. The disulphide bridge (----) was reduced and alkylated under the conditions of partial reduction used to prepare heavy chains. (b) The positions of the arginine residues in the fragment are shown. The numbering of the residues from 1-94 corresponds to the amino acid sequence in Mole *et al.* (1971) and the positioning of the arginine residues at 170 and 176 is from Fruchter *et al.* (1970).

Location of the label in affinity-labelled antibody

Separation of heavy and light chains. Each of the labelled antibody preparations was reduced, alkylated with iodoacetic acid and the chains separated on a column of Sephadex G-100 in 1M-propionic acid. The yield of light chains varied from 21-25% by weight and the amounts of the label associated with the chains is given in Table 1. Since the heavy chains contained the greater proportion of the label (labelling ratio of heavy/light chains = 3.3-5.0) further work was confined to the heavy chains.

Fragmentation of labelled heavy chains. In a previous experiment with affinity-labelled anti-Nap antibodies (Fleet *et al.*, 1972) two small labelled peptides were isolated from a tryptic digest of the CNBr fragment C-1 (Fig. 1a). These peptides accounted for 13% of the label on the antibody and the remainder of the label was associated with larger peptides, but it was not possible to isolate any pure labelled peptides from this fraction. It was assumed that this was because the label was in the hypervariable region of the heavy chain so that tryptic digestion gave rise to a large number of different labelled peptides.

In the present study therefore we aimed first to define the regions of the heavy chain that are labelled, by using methods that give large fragments whose position in the sequence is known. It was assumed that the presence of Nap derivatives on peptides of

30 residues or more would not affect their gel-filtration properties. Accordingly, CNBr cleavage was the first fragmentation method used, and this gives rise to fragments C-1, C-3, C-4, C-5 and C-6 (see Fig. 1a). Total reduction and alkylation of fragment C-1 releases two *N*-terminal fragments in low yield, due to partial methionine residues at positions 34 and 79. The second method used was citraconylation of the lysyl residues of fragment C-1, followed by tryptic digestion at the arginyl residues. There are arginine residues at positions 38, 66, 94, 170 and 176 (Fig. 1b) in the C-1 fragment from heavy chains of allotype Aa 3, so that from a tryptic digest of citraconylated reduced and alkylated C-1 fragment the peptides 1-38, 39-66, 67-94, 95-170, 171-176 and 177-251 can be separated as described by Mole *et al.* (1971). In addition, owing to the partial methionine residues at positions 34 and 79, the peptides 35-38 and 80-94 can also be isolated from the tryptic digest.

CNBr cleavage. The heavy chains from each of the three labelled antibody preparations (Table 1) were cleaved by treatment with CNBr, freeze-dried or dialysed against 6M-urea-0.2M-sodium formate, pH 3.3, and fractionated on a column of Sephadex G-100 in the same buffer. Dialysis of the CNBr cleavage products before gel filtration resulted in the loss of about 40% of the radioactivity; alternatively, when the cleavage products were freeze-dried before gel filtration, 30-35% of the radioactivity was eluted in the salt peak. Analysis of the material from the

salt peak showed that there were no amino acids associated with the ^3H radioactivity. However, to establish whether the radioactivity had been released by cleavage of an acid-labile bond during the CNBr reaction in 70% (v/v) formic acid, or alternatively whether it represented non-covalently bound Nap derivative released by the denaturing effect of the formic acid and/or 6M-urea, the effect of denaturation in 6M-guanidinium chloride at neutral pH was investigated. Labelled heavy chain from preparation 1b, containing 0.5 mol of label/mol of heavy chain, was totally reduced in 6M-guanidinium chloride with 0.05M-dithiothreitol, pH 8.0, at 37°C for 3h, alkylated with iodoacetic acid and chromatographed on a column of Sephadex G-25 in 6M-guanidinium chloride, pH 7.8. The reduced heavy chain was eluted at the void volume, but 30% of the ^3H radioactivity was eluted at twice the void volume. It seemed most unlikely that denaturation at neutral pH could have caused cleavage of a covalent bond, and in a further experiment with labelled antibody it was found that ϵ -Nap-lysine at 150 times the concentration was able to displace 6% of the ^3H radioactivity in 17 days at 2°C in the dark, and in 80 days 18% of the radioactivity was displaced. It is assumed therefore that the 40% of ^3H radioactivity released from the CNBr-treated material represents a Nap-lysine derivative bound non-covalently to the antibody. This radioactivity is therefore not included in Table 2, which shows the distribution of the ^3H radioactivity among the other fractions obtained by treatment of the heavy chain with CNBr. Fragment C-1, positions 1–251 of the heavy chain, contains the bulk of the label. The fragments C-3 and C-4 are not labelled. The significance of the very low amount of radioactivity in fraction C-5 is discussed later. Fraction C-6 contained the C-terminal octadecapeptide, positions 428–445, which was isolated in pure form by gel filtration on a column of Sephadex G-50 in 0.05M-NH₃. The radioactivity in fraction C-6 was not associated with the peptide, position 428–445; 50% of it was eluted

before the peptide but there was insufficient material for characterization. The remainder of the radioactivity was eluted in the salt peak.

In the case of heavy-chain preparations 1a and 1b, ^3H radioactivity was eluted between fractions C-3 and C-4 (C-3/4). This is presumably associated with a fragment from the variable region of the heavy chain, produced in low yield, since the constant region is accounted for and its sequence has been reported. Fragment C-4 consists of two fragments C-4a and C-4b of 60 and 17 residues respectively held together by a disulphide bond (see Fig. 1a). The fraction C-3/4 was totally reduced and alkylated with iodo[2- ^{14}C]acetic acid and fractionated on a column of Sephadex G-100 in 6M-urea–0.2M-sodium formate, pH 3.3. The reduced fragments C-3, C-4a and C-4b were resolved from each other and from the ^3H radioactivity, the latter was eluted from the column between fragments C-3 and C-4a. However, in the fraction containing the ^3H radioactivity, there was also a fragment of 78 residues consisting of fragments C-4a and C-6 (positions 368–445) which had arisen due to non-cleavage of the methionine residue at position 427 in a small percentage of molecules. Since neither fragment C-4a nor fragment C-6 were labelled, the ^3H radioactivity must be on a fragment of a similar size, but presumably from the variable region. It is possible, in fact, that the radioactivity is on the fragment 1–79, which was obtained in a yield of 19% on total reduction of fragment C-1, see below. It was not, however, possible to separate and characterize the labelled material.

The C-1 fragments of preparations 1b and 2 were totally reduced, alkylated with iodo[2- ^{14}C]acetic acid (O'Donnell *et al.*, 1970), and again fractionated on a column of Sephadex G-100 in 6M-urea–0.2M-sodium formate, pH 3.3, to separate fragments 1–34 and 1–79 (Fig. 1). The distribution of ^3H label among the three fragments is shown in Table 2. Reduced C-1 fragment is a mixture of molecules 1–251, 35–251 and 80–251, which were not resolved on the Sephadex G-100

Table 2. *Distribution of radioactivity among CNBr fragments of heavy chains*

The fragments were isolated by chromatography on a column of Sephadex G-100 in 6M-urea–0.2M-sodium formate, pH 3.3. Fragments C-1, C-3, C-4, C-5, C-6 and C-3/4 were produced by the reaction of CNBr with heavy chains and reduced fragments C-1, 1–79 and 1–34 were produced by total reduction and alkylation of C-1 fragment. In columns 1–6 the radioactivity is expressed as a percentage of that on the heavy chains and in columns 7–9 it is a percentage of the radioactivity of fragment C-1.

Preparation of antibody	Fragments	...	^3H Radioactivity (%)								Reduced	
			C-1	C-3	C-4	C-5	C-6	C-3/4	C-1	1–79	1–34	
1a			76	0	0	1	4	20	100	Not done	Not done	
1b			74	0	0	1	2	23	85	9	6	
2			93	0	0	1	7	0	76	11	13	

Table 3. *Distribution of radioactivity among tryptic peptides.*

The fractions were obtained by chromatography on a column of Sephadex G-50 in 50% formic acid of a tryptic digest of reduced, alkylated and citraconylated fragment C-1. The peptides contained in each of the fractions were identified as described by Mole *et al.* (1971) and the radioactivity is expressed as a percentage of the total radioactivity of the reduced C-1 fragment.

Preparation	Fraction Peptides in the fraction	³ H Radioactivity (% of total)			
		1 T2 (177-251) T3 (95-170)	2 1-38 39-66 67-94	3 80-94	4 35-38 171-176 91-94*
1a		40	40	10	10
1b		60	22	9	9
2		60	40	<1	Not counted

* Peptide 91-94, is the product of partial digestion by trypsin at tyrosine position 90 (Mole *et al.*, 1971).

column. A peptide, position 1-38, can be separated from a tryptic digest of the 1-79 fragment, and 70% of the ³H label is associated with this peptide (see below).

Tryptic digestion of reduced and alkylated C-1 fragment. Citraconic anhydride was used to block the lysine residues before digestion with trypsin, which cleaved the fragment at the 5 arginine residues shown in Fig. 1(b). After removal of the blocking groups at pH 3.5 the digest was fractionated on a column of Sephadex G-50 in 50% (v/v) formic acid (Mole *et al.*, 1971). Table 3 shows the distribution of ³H radioactivity among the four fractions obtained and the constituent peptides of each fraction. Fraction 1, consisting of peptides T2 and T3, was further fractionated on a column of DEAE-Sephadex A-25 with a linear gradient of NH₄HCO₃, pH 8.5, from 5 to 500 mM. Radioautography of the chymotryptic-tryptic digest of portions of the column eluate was carried out to characterize T2 and T3 peptides by their ¹⁴C-labelled *S*-carboxymethylcysteine-containing peptides (O'Donnell *et al.*, 1970). All the ³H radioactivity was associated with peptide T3. Fraction 2 was fractionated further on a column of Sephadex G-50 in 0.05 M-NH₃ and, by using the ¹⁴C radioautography method again it was found that at least 70% of the ³H radioactivity was associated with the 1-38 residue peptide. Fraction 3 from preparations 1a and 1b contained 10% of the ³H radioactivity of fragment C-1. The peptide, position 80-94, was separated from this fraction by gel filtration on a column of Sephadex G-50 in 0.05 M-NH₃ (Mole *et al.*, 1971), but the amount of ³H radioactivity associated with it was only 15% of the radioactivity in fraction 3, 70% of the radioactivity being of very low molecular weight. Fraction 4 also contained about 10% of the ³H label of reduced C-1 fragment from preparations 1a and 1b. This was investigated further by paper electrophoresis at pH 3.5, to see whether the small

labelled peptides (92-94 and 91-94) isolated by Fleet *et al.* (1972) were present. However, 32% of the ³H radioactivity was identified as free [³H]lysine and 25% of the radioactivity had the same mobility as ϵ -Dnp-lysine at pH 6.5 and 3.5, and was most likely a photolysis product of Nap-lysine. About 15% of the radioactivity was basic at pH 3.5 and neutral at pH 6.5 and could therefore have been the peptide Phe-CmCys-Y-Arg (91-94) isolated by Fleet *et al.* (1972), although there was insufficient material to establish this. However, the overall yield would not have been more than 1% of the total label on the heavy chain. The other peptide isolated by Fleet *et al.* (1972) X-Ala-Arg (92-94) was definitely not present, since there was no ³H radioactivity with the appropriate mobility at pH 6.5. The two small peptides that were isolated from fraction 4, positions 171-176 and 35-38, were not labelled. These peptides are basic at pH 6.5 and since there was no basic material at pH 6.5 with radioactivity, these two regions of the heavy chain could not have been labelled.

These results with the fragments obtained by CNBr cleavage, reduction and alkylation and tryptic digestion, enabled us to deduce the distribution of ³H label on C-1 fragment. Peptide, position 80-94, was isolated from fraction 3 (preparation 1b) in a comparable yield with that of the 1-79 fragment, as would be expected, but the radioactivity of peptide 80-94 corresponded to only 1 mol of label/160 mol of peptide, whereas fragment 1-79 was labelled to the extent of 1 mol of label/19 mol of fragment. The fragment 1-34 had radioactivity corresponding to 1 mol of Nap label/15 mol of peptide and the tryptic peptide T3 (position 95-170) was labelled to the extent of 1 mol of label/15 mol of peptide. The only part of the variable region about which information is lacking is positions 39-79. The fragment C-5, which comprises positions 35-79 and arises from molecules having methionine at both position 34 and 79, was obtained in very low

Table 5. *Amino acid composition of the N-terminal CNBr-cleaved fragment (position 1–34)*

The composition of the fragment isolated from non-immune Aa3 allotype heavy chain (Wilkinson, 1969) is compared with the same fragment isolated from labelled heavy-chain preparations 1b and 2. The analysis is based on Tyr+Phe = 3.0 (see the sequence in Fig. 3).

Amino acid	Composition (mol of amino acid/mol of fragment)		
	Non-immune	Preparation 1b	Preparation 2
Lys	1.0	1.0	1.4
Arg	0.2	0.6	0.7
Asp	1.6	1.9	1.5
Thr	2.9	3.2	3.2
Ser	4.9	5.1	5.3
Glu	3.9	4.0	3.5
Pro	1.4	1.3	1.4
Gly	4.8	4.7	4.4
Ala	1.7	2.3	1.9
Val	1.6	1.6	1.6
Ile	0.3	0.5	0.5
Leu	3.8	4.0	3.8
Tyr	1.4	1.2	1.4
Phe	1.6	1.8	1.6
CmCys	1.0	1.0	1.0
Hse	1.0	1.0	1.0

reason for this is not known. However, from the results of digestion of the fragment with carboxypeptidases A and B, discussed in the next paragraph, it seems likely that there is a variant for asparagine at position 28, which has not yet been identified. Assuming that the labelled fragment is digested with chymotrypsin at the same positions as the unlabelled fragment, then the implication of these results is that the label is located between positions 29 and 34. The very low percentage of ^{14}C radioactivity in fraction D did not migrate on electrophoresis with the ^3H radioactivity, so that the labelled peptides in fraction D are unlikely to be derived from positions 21–27.

Fig. 2 shows the distribution of ^3H radioactivity relative to the migration of the unlabelled peptides on electrophoresis of fraction D at pH 1.9. About 56% of the radioactivity was distributed over the same range of mobilities (–0.1 to +0.34) as the non-labelled peptides, and 35% migrated more slowly. The effect of the Nap-lysine derivative on the mobility of small peptides at pH 1.9 is difficult to predict, since the size and hydrophobicity of the group would tend to retard the peptide but the group carries a net positive charge at pH 1.9, which would increase the mobility. The slowly migrating material which had ^3H radioactivity (mobilities –0.5 to –0.15, Fig. 2) was analysed and the

Table 6. *Isolation of peptides from the chymotryptic digest of the N-terminal fragments 1–34*

Elution volumes (with 0.05M-NH₃, from Sephadex G-50) are given relative to exclusion volume = 1.0. The percentage of radioactivity found in each fraction is shown for the digestion of the fragment derived from the antibody preparations 1b and 2. Mobilities are given relative to ϵ -Dnp-lysine = 0, lysine = +1.0.

Fraction	Elution vol.	Preparation	Radioactivity (% of total)		Electrophoresis		Sequence implied from composition and known sequence (Wilkinson, 1969)	Position in fragment
			^{14}C	^3H	pH	Mobility		
A	1.3	1b	3	3	6.5	–0.61	$\begin{matrix} \text{Glu} < \text{---Ser} \\ \text{Glu-Gln} \end{matrix} \text{Leu-Glu-Glu-Ser-Gly-Gly-}$	1–18
		2	5	5				
B	1.6	1b	85	15	6.5	–0.33	$\begin{matrix} \text{Asp} \\ \text{Val} \end{matrix} \text{-Leu-Val-Lys-Pro-Gly-Ala-Ser-Leu}$	21–27
		2	88	20				
					6.5	–0.22	Thr-CmCys-Thr-Ala-Ser-Gly-Phe-Asn	21–28
					6.5	–0.18	Thr-CmCys-Thr-Ala-Ser-Gly-Phe-Asn-Leu	21–29
C	2.0	1b	4	1	3.5	+0.10	$\begin{matrix} \text{Gly} & \text{Phe} \\ \text{Ala-Ser-Ser-Tyr} \end{matrix}$	29–33
		2	4	10				
D	2.2	1b	7	81	3.5	+0.33	$\begin{matrix} \text{Thr-Leu} \\ \text{Gly-Ser-Ser-Phe-Tyr} \end{matrix}$	19–20
		2	3	65				
					3.5	+0.14	$\begin{matrix} \text{Ser-Ser-Phe-Tyr} \\ \text{Tyr} \end{matrix}$	30–33
					1.9	–0.10		
					3.5	+0.14	Ser-Ser-Tyr	30–32
					1.9	+0.11		
					3.5	0.0	Tyr	33
					1.9	+0.14	Tyr-Hse	33–34
					3.5	+0.14		
					1.9	+0.21	Hse	34
					3.5	0.0		
					1.9	+0.34		

amino acid residues present were serine, glycine, alanine, tyrosine, phenylalanine and homoserine, but in amounts tenfold in excess of the amount of label present; this was no doubt due to some adsorption of the large excess of unlabelled peptides that had

Fig. 2. Migration of labelled peptides from fraction D of a chymotryptic digest of the N-terminal fragment, position 1-34, on electrophoresis at pH 1.9

The migration of the labelled peptides, indicated by the ^3H radioactivity, was detected by eluting strips of paper with 0.1 M-acetic acid from the origin to the position of migration of lysine. The positions of the unlabelled peptides in the digestion mixture were detected by staining a narrow strip with ninhydrin. The eluted peptides were analysed (see Table 6 and the text). Mobilities are relative to ϵ -Dnp-lysine = 0 and lysine = +1.0.

migrated across the paper, but it prevented any conclusion being drawn as to the composition of the labelled peptides.

The reasons for the failure to isolate labelled peptides are (a) the multiple positions of chymotryptic cleavage between positions 29 and 34, and (b) the heterogeneity of sequence; also if more than one position in this region is labelled, chymotryptic digestion would give rise to more labelled peptides than unlabelled ones, as is found to be the case (Fig. 2).

To obtain more evidence for the presence of ^3H label on residues 29-34, the 1-34 residue fragment was digested with carboxypeptidases A and B together (enzyme/substrate ratio, 1:30, by weight of each enzyme) at 37°C and pH 7.5 for 36 h. A sample of the digest was analysed and the digest chromatographed on a column of Sephadex G-50 in 0.05 M- NH_3 . Two fractions were obtained; fraction α contained 91% of the ^{14}C radioactivity and fraction β contained 78% of the ^3H radioactivity eluted from the column. Table 7 shows the analyses of the whole digest and of fraction α ; the latter analysis was based on a value of 1.3 for lysine, since no lysine was released by the enzymes (see analysis of fraction β in Table 7). It is apparent by comparison of the analyses for tyrosine and phenylalanine and the sequence (Fig. 3) that phenylalanine at position 27 has been digested in 70% of the molecules; but from the analyses for glycine, serine and alanine, digestion beyond this point must be minimal. A comparison of the residues released by the enzyme with the sequence of the fragment, suggests that there are variants in the sequence at the C-terminal end which have not yet been identified. In particular the amount of aspartic

Table 7. Amino acid composition of the products of digestion of fragment 1-34 with carboxypeptidases A and B

Fractions α and β were derived by fractionation of the digest on a column of Sephadex G-50 in 0.05 M- NH_3 . Analysis of the whole digest is based on Tyr+Phe = 3.0. Analysis of fraction α is based on Lys = 1.3, since no lysine was removed by the digestion. The analysis of fraction β was based on Hse = 1.0.

Amino acid	Whole digest	Fraction α	Whole digest -fraction α	Fraction β
Lys	1.3	1.3	0	0
Arg	0.4	0.4	0	0
Asp	1.6	1.3	0.3	0.3
Thr	3.0	2.5	0.5	0.6
Ser	5.0	4.0	1.0	1.8
Glu	3.6	4.2	0	0
Pro	1.3	1.3	0	0
Gly	5.6	5.4	0.2	0.6
Ala	2.0	1.7	0.3	0.4
Val	1.6	1.5	0.1	0
Ile	0.5	0.2	0.3	0.2
Leu	4.0	3.8	0.2	0.6
Tyr	1.3	0.1	1.2	1.7
Phe	1.7	0.3	1.4	1.3
CmCys	1.0	1.0	0	0
Hse	0.8	0	0.8	1.0

Fig. 3. Amino acid sequence of the N-terminal fragment, positions 1–34

The amino acid sequence of rabbit heavy chain of allotype Aa3 is from Wilkinson (1969) with an additional variant at position 29, deduced from the composition of peptides isolated from a chymotryptic digest (see Table 6 and the text).

acid (or asparagine) is less than expected from the sequence and, on the other hand, threonine, which does not occur in the reported sequence between positions 27–34, was released by the carboxypeptidases. If it is assumed that labelled molecules are digested to the same extent as unlabelled fragment, since 78% of the ^3H radioactivity was removed from the peptide by the combined carboxypeptidases, the labelled residues must be located within section 27–34. An attempt was made to separate the labelled residues by paper electrophoresis at pH 1.9, but although about 50% of the ^3H radioactivity migrated more slowly than the unlabelled amino acids, recovery of the labelled residues was too low to allow further characterization.

***N*-Terminal 1-79 fragment.** A partial sequence for the C-5 fragment, position 35-79 of the Aa3 heavy chain, was reported by Mole *et al.* (1971) and from this it was anticipated that tryptic digestion would release a large peptide, position 1-38, and smaller peptides, including the high-yield peptides, positions 39-43, 65-66 and 67-71. The fragment was digested with trypsin (enzyme/substrate ratio 1:100, by weight) at pH 8.1 for 2½ h at 37°C and fractionated on a column of Sephadex G-50 in 0.05M-NH₃. The fraction containing the peptide 1-38 was detected by counting the eluate for ¹⁴C radioactivity and confirmed by the method of radioautography of chymotryptic-tryptic peptides of portions of the eluate (O'Donnell *et al.*, 1970). In this way it was estimated that at least 70% of the ³H label was eluted with the *N*-terminal 1-38 residue peptide. The other tryptic peptides listed above were isolated and found to be unlabelled. The two regions of the fragment 44-64 and 72-79 were not isolated, and the 30% of ³H radioactivity not accounted for on the 1-38 residue fragment may be associated with these regions. The *N*-terminal peptide, position 1-38, was digested with chymotrypsin (enzyme/peptide ratio 1:20, by weight) at pH 8.0 for 3 h at 37°C and fractionated on a column of Sephadex G-50 in 0.05M-NH₃. Four fractions were separated

corresponding to the four fractions isolated from the chymotryptic digest of the 1-34 fragment (Table 6) and the ^3H radioactivity was confined to fraction D. Paper electrophoresis at pH 1.9 of this fraction was carried out and the peptides corresponding to positions 29-33 were isolated and in addition the dipeptide valylarginine (position 37-38) was also isolated. The distribution of ^3H was similar to that found for fraction D from the digest of the 1-34 fragment (Fig. 2), with 54% migrating with the same mobility as the non-labelled peptides and 30% migrating slower than ϵ -Dnp-lysine (mobility = 0).

Peptide T3. This peptide extends from position 95 in the variable region to 170 in the constant region of the heavy chain (see Fig. 4). The sequence of the constant region from position 115 to 170 has been published (Cebra *et al.*, 1968; Fruchter *et al.*, 1970) but attempts to determine the sequence of the *N*-terminal section of peptide T3 have been unsuccessful. This is assumed to be due to a very high degree of variability in sequence and evidence for this has been presented (Fruchter *et al.*, 1970; Mole *et al.*, 1971); also this region of the rabbit heavy chain from position 95 is homologous to the hypervariable region in human γ 1 chain, positions 101–107 (Kehoe & Capra, 1971). It was assumed that the label would be within the variable portion of the peptide, and two approaches were used in an attempt to locate the ^3H label. On the one hand, the tryptic and chymotryptic peptides of the constant region of peptide T3 were isolated and at the same time ^3H -labelled peptides were sought. The second approach was to degrade the peptide from the *N*-terminal end both enzymically and chemically.

Tryptic digestion of peptide T3 (95–170). Peptide T3 (2.5 μmol) isolated from antibody preparation 1a, of which 0.11 μmol carried the Nap label, was digested with 1.2 mg of trypsin (enzyme/peptide ratio 1:20, by weight) at pH 8.3 for 1½ h at 37°C. There are three lysine residues in peptide T3 and tryptic digestion at lysines 123 and 149 in the constant region of the heavy

Fig. 4. Peptide T3, positions 95–170

The positions of the two lysine residues in the constant part of the peptide are shown by vertical bars. A third lysine residue occurs fractionally in several positions within the variable part of peptide T3, positions 95–114 inclusive. Ts3 and T-T3-LY are peptides isolated from a tryptic digest of peptide T3. The numbering from 115–170 is from the amino acid sequence of this section (Fruchter *et al.*, 1970).

chain (Fig. 4) gives rise to two peptides, Ts3 (124–149) and T-T3-LY (150–170). These two peptides were isolated by fractionation of the tryptic digest of peptide T3 on a column of Sephadex G-50 in 0.05 M-NH₃ and by paper electrophoresis as described by Cebra *et al.* (1968). The two peptides which moved as discrete bands on paper electrophoresis, were in each case resolved from the ³H radioactivity and were obtained pure with less than 1 mol of label/500 mol of peptide.

The third lysine in peptide T3 is in the variable region, positions 95–114, but since no lysine-containing peptides could be isolated from the tryptic digest, other than Ts3 and T-T3-LY, this lysine must occur fractionally at many different positions, thus giving rise to many different tryptic peptides from the variable region of peptide T3. The ³H radioactivity in the tryptic digest migrated on electrophoresis with other unidentified peptide material over a wide range of mobilities and it was not possible to recover sufficient labelled material for characterization. Since peptides Ts3 and T-T3-LY contained respectively 26 and 21 residues each, if either of them had been labelled, the position of the label within the peptides would not have affected their electrophoretic mobility and discrete bands of radioactivity would have been detected on paper electrophoresis. The spread of radioactivity actually observed when fractions of the tryptic digest containing label were subjected to electrophoresis is taken as evidence that the label is in the variable region of peptide T3.

Combined tryptic-chymotryptic digestion of peptide T3. The *N*-terminal portion of the constant region (positions 115–123) is not accounted for in the two tryptic peptides Ts3 and T-T3-LY. A peptide from this section was isolated from a chymotryptic-tryptic digest (150 μg of each enzyme/μmol of peptide in 0.05 M-NH₄HCO₃ buffer, pH 8.0, at 37°C for 4 h) by fractionation on a column of Sephadex G-50 in 0.05 M-NH₃ (elution volume 2.0 relative to the void

volume) and by electrophoresis at pH 3.5 (mobility 0.5 relative to lysine = 1.0). The peptide had the partial sequence Val-Thr-Val-(Ser,Ser,Gly,Glx,Pro)-Lys, and comes from position 115–123 (Fruchter *et al.*, 1970; D. Pratt, unpublished work). The radioactivity of the fraction from which this peptide was isolated migrated over a wide range of mobilities and it was not possible to isolate any labelled peptides. It seems unlikely therefore that the constant region, positions 115–123, was labelled. The implication of the foregoing results is that the Nap label is located within the *N*-terminal portion of peptide T3, between positions 95 and 114. To substantiate this, degradation of the peptide from the *N*-terminal end was attempted.

Amino-peptidase M digestion of peptide T3 (95–170). Peptide T3 was digested with amounts of enzyme from 2500–15000 munits/μmol of peptide (in 0.1 M-NH₄HCO₃ buffer, pH 8.0, at 37°C for 24 h) and the digests were fractionated on a column of Sephadex G-50 in 0.05 M-NH₃ into three fractions, undigested T3, partially digested T3 and free amino acids. The extent of the digestion could be followed by determining the ¹⁴C radioactivity of the fractions, since the *S*-carboxymethylcysteine residue at position 146 (52 residues from the *N*-terminal end of peptide T3) is labelled with ¹⁴C radioactivity. About 45% of the ³H radioactivity could be released by the amino-peptidase but at the same time 50% of the molecules of peptide T3 had been completely digested down to their constituent amino acids. Conditions could not be found where the label was released without extensive degradation of some of the molecules occurring and it was apparent that the enzyme digested the peptide, molecule by molecule, down to its constituent amino acids and since only one in 14 of the molecules was labelled, it was impossible to infer the location of the label from these experiments.

Automatic sequential Edman degradation of peptide T3 (95–170). Peptide (0.41 μmol) derived from preparation 2, was subjected to 30 steps of sequential

Edman degradation in a Beckman Sequencer model 890B. The thiazolinone amino acids, extracted into chlorobutane after each step, were counted for ^3H and ^{14}C radioactivity. Only 5% of the ^3H radioactivity and 1.5% of the ^{14}C radioactivity was extracted into the chlorobutane in the first ten steps of the degradation. Extraction of the ^{14}C radioactivity is assumed to indicate extraction of the whole peptide into the solvent phase. In the next 20 steps of degradation (11–30) only a further 1% of ^3H radioactivity and 1.5% of ^{14}C radioactivity were extracted into the chlorobutane. However, when the residue in the cup after 30 degradation steps was counted for radioactivity, only 15% of both ^{14}C and ^3H counts were present. Therefore 80% of the peptide must have been lost in the benzene and ethyl acetate extracts used to remove the reaction products between each step so that it was impossible to interpret the results. However, as some ^3H radioactivity was cleaved off in the first ten steps of the degradation, it was decided to attempt a manual Edman degradation, in which it would be possible to measure the radioactivity present before each step as well as the amount cleaved off.

Manual Edman degradation of peptide T3 (95–170). In view of the loss of peptide encountered in the automatic sequencer, extraction of reagents and reaction products before cleavage with trifluoroacetic acid was omitted. Coupling was performed with phenylisothiocyanate in a buffer of *N*-ethylmorpholine-pyridine-acetic acid, pH 8.5, and after cleavage with trifluoroacetic acid the thiazolinone was extracted into benzene. The total radioactivity extracted into benzene for five degradation steps was 7.5% of the ^3H and 2.5% of the ^{14}C radioactivity. Edman degradation was performed up to eight steps, but in the last three steps equivalent amounts of ^{14}C and ^3H were extracted. The small amount of ^3H in excess of ^{14}C radioactivity extracted in the first five steps of degradation may be taken as evidence in support of the conclusions, drawn from the digestions of peptide T3 with trypsin and chymotrypsin, that the Nap label is located at the *N*-terminal end of peptide T3. The failure to remove more than a small percentage of the label by the Edman-degradation method may be due to steric hindrance, resulting from the reaction of the phenylisothiocyanate with the Nap-lysine derivative.

In conclusion, the position of the label in peptide T3 could not be definitively proved, but such evidence as was obtained from tryptic and chymotryptic digestions supported the assumption that it would be located within the variable portion of the peptide, i.e. between positions 95 and 114.

Discussion

The specificity of covalent binding of ϵ -Nap- ^3H -lysine in the hapten-binding site was demonstrated by the fact that the labelled antibody was not bound

to the Dnp-lysyl-Sepharose immunoabsorbent column, used in the isolation of the antibody from serum. Also, by equilibrium dialysis against ϵ -Nap- ^3H -lysine, the valency of labelled antibody with 1.5 mol of ϵ -Nap-lysine covalently bound/mol, was found to be only 0.4. The anti-'Dnp' antibody was also shown to be specifically labelled, both by passage through the Dnp-lysyl-Sepharose column and by equilibrium-dialysis studies. This demonstration of specificity of labelling was particularly important in the latter case, since the affinity constant for ϵ -Nap-lysine of the anti-'Dnp' antibody was only $5 \times 10^5 \text{ M}^{-1}$ at 4°C , compared with anti-Nap antibody for which the affinity constant varied from 5×10^6 to $1.2 \times 10^7 \text{ M}^{-1}$ at 4°C .

Kiefer *et al.* (1970) described the use of two aryl azides, 4-azido-2-nitrobenzyltrimethylammonium and 4-azido-2-nitrobenzyltriethylammonium to photoaffinity-label acetylcholine-binding sites on membranes. They reported the irreversible inactivation of acetylcholinesterase on erythrocyte membranes by photolysis in the presence of the two reagents. However, in a subsequent paper (Singer *et al.*, 1973) they showed that with a 1000-fold excess of *p*-aminobenzoate, the enzyme was protected from inactivation by photolysis in the presence of the aryl azides. The authors stated that *p*-aminobenzoate was acting as a scavenger to remove reactive nitrene present in free solution and concluded therefore that specific photolabelling of the enzyme's active site was not being achieved. In view of these results, we photolysed the complex of anti-Nap antibody (25 μM) and ϵ -Nap- ^3H -lysine (50 μM) in the presence of up to 50 mM *p*-aminobenzoate and found that there was only a 15% decrease in the extent of labelling. Irrespective of the mechanism operating in the experiments described by Singer *et al.* (1973) with *p*-aminobenzoate, our studies leave us in no doubt that we have specific labelling of the antibody-binding site.

There is evidence from the work of Parker & Osterland (1970) that the antibody-binding site includes a hydrophobic region that contributes to the binding energy; they reported that the hydrophobic compound 8-anilino-naphthalene-1-sulphonate would bind to non-immune immunoglobulin and to various antibodies with affinities ranging from $1 \times 10^3 \text{ M}^{-1}$ to $1 \times 10^4 \text{ M}^{-1}$, the affinities being greater for anti-Dnp antibodies than for other immunoglobulins. It was not therefore surprising to find that if 2 mol of ϵ -Nap- ^3H -lysine were photolysed in the presence of 1 mol of non-immune immunoglobulin covalent binding occurred to the extent of 0.3–0.4 mol of label/mol of immunoglobulin, although the association constant of Nap-lysine for non-immune immunoglobulin was only $1 \times 10^3 \text{ M}^{-1}$ at 4°C . However, when 2.5 mol of ϵ -Nap- ^3H -lysine and 1 mol of non-immune immunoglobulin were incubated together in the dark, and then passed down a column of Sephadex G-25 at neutral

pH, the immunoglobulin was eluted at the void volume with less than 0.02 mol of label/mol. This latter procedure was followed as a routine in the affinity labelling of antibody, as described in the Results section, to ensure that, if there was any denatured or very-low-affinity antibody in the antibody preparation, it would not be labelled. Parker & Osterland (1970) showed that the binding of 8-anilino-naphthalene-1-sulphonate was predominantly in the Fab fragments, and in our studies with non-immune immunoglobulin, covalently labelled as described above, the Nap label was predominantly on the heavy chain (3.9:1, heavy/light chain) and after reaction with CNBr the label was on the C-1 fragment. Also the fragments 1-34 and 1-79, isolated after total reduction of C-1 fragment, contained 10 and 11% of the label respectively. Although not conclusive, these results strongly support the notion that the non-specific hydrophobic binding site is in the same region of the molecule as the specific antibody-binding site.

The method of affinity labelling was pioneered by Singer and his colleagues (Wofsy *et al.*, 1962) who used diazonium reagents to label the binding site of anti-hapten antibodies. The method has been extensively used and tyrosine has been identified as the labelled residue in the majority of cases. Location of the labelled tyrosine and, by implication, of the antibody-binding site is, however, a difficult task with heterogeneous antibodies especially when the labelled residues are in the hypervariable regions. Thorpe & Singer (1969) isolated labelled dipeptides, which could be placed in the variable-region sequences of heavy and light chains of both rabbit and mouse antibodies; one such peptide, Thr-Tyr, occurs in rabbit heavy chains at positions 89-90, which is just *N*-terminal to the intrachain cysteine residue at position 92. Franek (1971, 1973) labelled pig anti-Dnp antibodies with *m*-nitrobenzenediazonium fluoroborate and reported that tyrosine residues in the lambda chains at positions 33 and 93 were labelled and also a tyrosine in the heavy chains between positions 22 and 36. Guinea-pig IgG 2 anti-Dnp antibodies have been labelled with *m*-nitrobenzenediazonium fluoroborate and the labelled residues identified are tyrosine residues in the heavy chains at positions 32 (and/or 33), 60 and between 99 and 119 (Ray & Cebra, 1972), which are all within the hypervariable regions. By using a paired iodination technique with rabbit anti-3-azopyridine antibody of limited heterogeneity, Friedenson *et al.* (1971, 1972) isolated two iodinated peptides, Val-Ser-ITyr and ITyr-Ser-Leu, which were most probably from the heavy-chain hypervariable region 95-114.

Strausbauch *et al.* (1971) used bromoacetyl reagents to label goat anti-Dnp antibodies and the residues labelled were identified as both lysine and tyrosine. The most noteworthy work with these re-

agents was with the myeloma protein MOPC 315, which binds ϵ -Dnp-lysine. This protein was labelled with a bifunctional bromoacetyl reagent that cross-linked the heavy and light chains labelling tyrosine at position 34 on the light chain and lysine at position 54 on the heavy chain (Givol *et al.*, 1971). This tyrosine on the light chain was also shown to be labelled with the diazonium reagent *m*-nitrobenzenediazonium fluoroborate (Goetzl & Metzger, 1970). Another mouse myeloma protein, TEPC 15, which binds phosphorylcholine, was affinity labelled with a diazonium reagent, and tyrosine in the same position on the light chain was found to be labelled (Chesebro & Metzger, 1972).

The Nap reagent has certain advantages over the diazonium and bromoacetyl reagents. These are (1) that the reactive group, the nitrene, can be generated photolytically in the binding site, (2) it is structurally very similar to the precursor and (3) it is non-selective and can in principle react with any C-H bond; consequently labelling occurs in the site, as has been amply proved. However, the non-selectivity of the reaction undoubtedly increases the problem of isolating labelled peptides, since there will be many different products of the reaction. This no doubt contributed to our inability to isolate labelled peptides even from the region 29-34 where at least some of the sequences are known. However, it may be argued that it is the location of the labelled residues that is important rather than their identity, and in the present studies we have shown that about 80% of the label on the heavy chain was in the two hypervariable regions, positions 29-34 and 95-114, with the possibility that 20% was in the hypervariable region 50-57. These positions correspond to three hypervariable regions in the human γ 1 heavy chains (Kabat & Wu, 1971). A fourth hypervariable region in human γ 1 chains was postulated by Kehoe & Capra (1971); the corresponding region in the rabbit heavy chain, positions 80-85, is not a hypervariable region, but is associated with a genetic marker (Mole *et al.*, 1971; L. E. Mole, personal communication) and this region was not significantly labelled. The light chain undoubtedly contributes to the binding site, but the more extensive labelling of the heavy chain presumably reflects the orientation of the hapten, and in particular of the azide group, in the binding site.

In the previous affinity-labelling studies with ϵ -Nap-lysine (Fleet *et al.*, 1972) two labelled peptides were isolated, accounting for 13% of the Nap label on the antibody and in which the label was on position 92 and 93 of the heavy chain. In the present studies a maximum of 1% of the label was associated with this region of the molecule. The antibody used in the previous studies was isolated from a pool of antisera raised in rabbits that were of mixed 'a' locus allotype, whereas in the present work we purposely selected rabbits that were allotypically homozygous, Aa3, to

eliminate allotype-related sequence variations. Since there are sequence differences between our Aa1 and Aa3 rabbits in positions 80–85, it is possible that the three-dimensional structure in the vicinity of the antibody site may be affected in such a way as to explain the different labelling pattern in the present study compared with that reported by Fleet *et al.* (1972).

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