Table 1. EFFECT OF INCREASED POLYMERIZATION OF BOVINE SERUM ALBUMIN ON THE TITRE OF A SERUM CONTAINING "INCOMPLETE" ANTI-D ANTIBODIES

Anti-D Reciprocal of titre	Serum albumin Untreated	Treated
1	+++	+++
2	++	+++
4	+	++
8	(+)	++
16	Weak	++
32	–	++
64	–	++
128	–	++
256	–	(+)
512	–	Weak
1,024	–	–

When bovine serum albumin preparations were treated with the bifunctional reagent N,N′-*p*-phenylene-*bis*-maleimide[6], electrophoresis revealed the expected increase in dimer and the formation of a trace amount of one higher polymer in most instances. The potentiating capacity of the albumin was not significantly affected by this treatment, suggesting that the higher polymers are implicated rather than the dimer.

These results establish firmly that the potentiation of "incomplete" Rh agglutinins by serum albumin preparations is a function of their polymer content. They indicate a useful procedure for increasing the sensitivity of the titration of "incomplete" Rh antibodies which has implications in relation to the standardization of reference antisera.

A patent application has been made in the UK covering this work, and enquiries should be made to the National Research Development Corporation. J. M. J. thanks the Medical Research Council for a grant while doing this work.

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Affinity Labelling of Antibodies with Aryl Nitrene as Reactive Group

THE covalent binding of a haptene to its specific antibody with a view to identifying the section of peptide chains which form the combining site has been pioneered by Singer *et al.*[1,2], who named this approach affinity labelling. Antibodies were prepared in rabbits against several aromatic haptenes such as benzene arsonate, and affinity labelling was performed by reaction with *p*-(arsonic acid)-benzene diazonium fluoroborate through the reactive diazonium group. The reaction with specific antibody was considerably more rapid than that with inert IgG because of the concentration of reagent in the combining site. Subsequent separation of heavy and light chains followed by enzymic digestion led to the isolation of one predominant dipeptide (Val-Tyr) from the light chain and another (Thr-Tyr) from the heavy chain; both of these were substituted on the tyrosine residue. No sequence data were available on the peptide chains of rabbit IgG, but from comparison with the known sequences of the light chains of human and mouse IgG it was suggested that the tyrosine residue 86 in the light chain may have been the one labelled.

One difficulty of this technique was that the reactive diazonium group was not itself likely to be a part of the specificity determining part of the haptene. Further, the diazonium group will react with only a limited number of amino-acids, particularly tyrosine, histidine and lysine, so that there was a possibility that reaction might occur with a residue relatively near, but not in, the site. With some antibody preparations no specific reaction occurred[3]. This risk was increased because only about 0·5 of the two antibody combining sites per molecule could be allowed to react before the non-specific reaction with inert IgG, or with antibody the specific sites of which were protected by haptene, became significant.

We have therefore attempted to devise an alternative method in which the precursor of the reactive species is stable during coupling to a protein and subsequent injection into rabbits, but which may be activated in mild conditions that do not cause dissociation or disruption of the antibody–haptene complex. The specificity determinants would thus include the reactive group. Further, a group was sought which could insert into a C—H bond and hence be capable of reacting with any amino-acid residue.

The only chemical species capable of insertion into carbon–hydrogen bonds are carbenes and nitrenes. These can be generated photochemically from diazo compounds (to carbenes) and azides (to nitrenes). Aliphatic diazo compounds are only stable when adjacent to a carbonyl or similar electron-withdrawing group, and photolysis of such a system gives a classical situation for the Wolff rearrangement to a ketene. (This rearrangement has been observed to dominate the covalency changes in photolysis of an enzyme-bound diazo compound[4].) Moreover, aliphatic diazo compounds decompose very rapidly in aqueous solution below *p*H 3 and they might not survive *in vivo* long enough to stimulate a specific antibody response. Acyl azides suffer from even more disadvantages: they are highly reactive; photolysis is only effective at wavelengths so short (300 nm) that aromatic residues in the protein would be destroyed by the incident radiation; and photolysis gives the Curtius or Schmidt rearrangement to isocyanate with consequent reduction in insertion products. Alkyl azides are also ruled out on grounds of instability and short ultraviolet absorption maxima.

Aryl azides, however, fulfil all the requirements above. These materials are likely to be stable indefinitely *in vivo*; with appropriate substituents their absorption maxima are well clear of protein absorption; photolysis to the aryl nitrene is smooth and efficient; and intramolecular rearrangement does not compete effectively with intermolecular abstraction and insertion processes.

We report here the isolation of a specific antibody against a phenyl azide determinant, the preparation of the antibody–haptene conjugate, and the photolysis of this conjugate. Photolytic generation of the nitrene at the binding site of the antibody leads to specific labelling of the antibody molecule.

The 4-azido-2-nitrophenyl (NAP) group (Fig. 1) was chosen as the antigenic determinant because neither of the *ortho* positions to the azide was occupied, and because this group could be conjugated to a protein via the ε-amino group of lysine by treatment of the protein with 4-fluoro-3-nitrophenyl azide (Fig. 1, if $R = F$).

4-Fluoro-3-nitroaniline was diazotized and subsequently treated with sodium azide at −20° C to give a 70 per cent

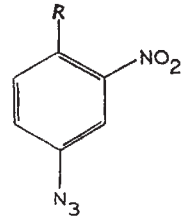

Fig. 1. The 4-azido-2-nitrophenyl group. If $R = F$ this structure represents 4-fluoro-3-nitrophenyl azide, and if $R = OH$ it is 4-azido-2-nitrophenol.

yield of 4-fluoro-3-nitrophenyl azide (m.p. 52° C) as light-sensitive straw coloured needles (from light petroleum, b.p. 40°–60° C). Treatment of this compound with aqueous methylamine gave 4-azido-(N-methyl)-2-nitro-aniline (m.p. 122°–123° C) which had an ultraviolet absorption maximum at 460 nm (ε 4,800).

The antigen was prepared in the dark because the NAP group is sensitive to light. Bovine γ-globulin (BGG 1 g) was dissolved in borate buffer, *p*H 9·8 (100 ml.), and the solution was treated with 4-fluoro-3-nitro-phenyl azide (1·0 g) in acetone (10 ml.). The reaction mixture was stirred at 40° C overnight and then filtered. The filtrate was dialysed against water for three days, filtered and then freeze-dried to give NAP–BGG (about 1 g) as a pink, light-sensitive product.

The number of NAP groups per mole BGG was determined by analysis of residual lysine residues, by measurement of absorption at 460 nm, and by measurement of N_2 evolution during irradiation. All three determinations indicated approximately 60 NAP-lysine residues/mole BGG.

Twelve rabbits were given a primary subcutaneous injection of 5 mg NAP–BGG in Freund's adjuvant. A booster injection was given intravenously either with 1 mg NAP–BGG in adjuvant or with 1 mg of alum-precipitated antigen; the second method gave a marginally better response. The animals were bled over a period of 4 months, with intermittent booster injections when assays of the sera indicated that they were needed. The antibody response was 400–800 μg/ml. of antibody precipitating with NAP-human serum albumin, and the antibody was therefore assumed to be specific for the haptenic group.

The antibodies were precipitated (according to the method of Eisen[5]) with either NAP–BGG or NAP–HSA and the resulting precipitate dissolved overnight at 37° C in a saturated solution of 4-azido-2-nitrophenol (Fig. 1, if R=OH) in sodium carbonate–sodium bicarbonate buffer, *p*H 9. The resulting solution was passed down a DEAE-cellulose column to remove antigen and haptene. (4-Azido-2-nitrophenol was prepared by treatment of 4-fluoro-3-nitrophenyl azide with sodium hydroxide.)

The isolated anti-NAP antibodies gave a single slow-moving electrophoretic band on cellulose acetate, a single symmetrical peak s_{20w}=6·3 on ultracentrifugation, and single line in double diffusion against goat anti-whole rabbit serum. These antibodies thus seem to contain only rabbit IgG molecules.

The radioactive haptene, ε-NAP-[4,5-$^3H_2$]L-lysine (NAP-[^{3}H]lysine) (Fig. 1; R=ε-amino-L-lysine) was prepared by treating a solution of 4,5-[$^3H_2$]L-lysine monohydrochloride in sodium carbonate–sodium bicarbonate buffer (*p*H 10·8) with an equimolar amount of 4-fluoro-3-nitrophenyl azide in acetone. The reaction mixture was stirred overnight at 40° C. After extraction with ether, NAP-[^{3}H]lysine was purified by thin-layer chromatography on silica gel plates developed with diethyl ketone-water.

The affinity constant of the anti–NAP antibody with NAP-[^{3}H] lysine as measured by equilibrium dialysis[6] was $6{\cdot}7 \times 10^6$ l./mole at 4° C.

Anti-NAP antibody (3 mg/ml.) was treated with a five-fold molar excess of NAP-[^{3}H]lysine at 4° C in 0·03 M phosphate buffer (*p*H 7·4) and left for 2 h in the dark; the mixture was then passed down a 'Sephadex *G*-25' column which had previously been equilibrated with 10^{-7} M NAP-[^{3}H]lysine in the same buffer, and the protein fraction collected. The protein had absorbed approximately 2 moles of NAP-[^{3}H]lysine per mole of antibody. The antibody–haptene conjugate thus prepared was irradiated at 4° C for 18 h by two Mazda 125 W *MB/V* pearl glass lamps immersed in a solution of sodium nitrite (to absorb any radiation of shorter wavelength than 400 nm).

IgG was added as carrier to an aliquot of the resulting reaction mixture and the protein was precipitated with trichloroacetic acid; the precipitate was washed and then dissolved in 0·1 M sodium hydroxide and the radioactivity in this solution determined. This method indicated that 1·1 moles of NAP-[^{3}H]lysine was bound per mole of protein. In a second experiment 1·2 moles haptene was bound per mole protein.

When the affinity constant of the reacted antibody for NAP-[^{3}H]lysine was remeasured, it was found to be too low to obtain a satisfactory figure. The association constant was certainly less than 10^4. The rather low value of bound haptene per molecule in unreacted antibody was probably a result of partial blocking of the sites with nonradioactive haptene during isolation of the antibody, and of the presence in the radioactive haptene of a small proportion of molecules which had been accidentally activated by light before the reaction. When radioactive NAP-lysine was diluted with a twenty-fold excess of nonradioactive haptene no significant radioactive labelling could be detected, and if inert rabbit IgG was used in place of antibody no labelling occurred. Hence it seems that the photogenerated nitrene is localized specifically within the antibody combining site.

After a total reduction with dithiothreitol in 6 M guanidine[7] the reacted antibody was dissociated into its peptide chains by chromatography on a 'Sephadex *G*-200' column in 5 M guanidine. The ratio of label was found to be 3·5/1·0 in the heavy/light chains respectively. In a preliminary experiment, treatment of the heavy chain with cyanogen bromide[8] showed no significant labelling in the peptides from the C-terminal half of the chain. It seems therefore that this is a satisfactory method of positioning a covalently bound reagent specifically in the part of the peptide chains which form an antibody combining site. Because most of the amino-acid sequence of the N-terminal half of the heavy chain of rabbit IgG has been determined[9] it should be possible to define the section or sections concerned.

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Production of the Influenza Syndrome in Man with Equine Influenza Virus

An antigenic relationship has been established between A/equi-2 influenza virus isolated in 1963 and the 1968 Hong Kong variant of human type A_2 influenza virus[1]. This relationship suggests that the Hong Kong variant was derived from the equine virus. To evaluate this relationship further and to test the possibility of an exchange of viruses between species we have carried out virus challenge studies with normal volunteers. Here we describe the occurrence of clinical syndromes similar to those described for naturally occurring influenza after inoculation of antibody-free volunteers with a strain of influenza A/equi-2 virus.