Immunology, 1966, **10**, 465.

Chemical and Serological Studies with an Iodine-Containing Synthetic Immunological Determinant 4-Hydroxy-3-iodo-5-nitrophenylacetic Acid (NIP) and Related Compounds

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(*Received 25th October* 1965)

Summary. The synthesis and properties of 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and several related compounds are described. Conjugates of NIP with proteins are prepared from the azide, synthesized from commercial 4-hydroxyphenylacetic acid. Sera from rabbits and mice immunized with NIP–ovalbumin or NIP–chicken serum globulin bind $N^{131}IP$-containing compounds, as judged from precipitation of radioactivity by salting-out of immunoglobulins. Homogeneous binding is obtained with $N^{131}IP$–polylysine, $N^{131}IP$–ε-amino-n-caproic acid ($N^{131}IP$–aminocap), and other structurally related haptens; non-homogeneous binding is obtained with $N^{131}IP$–bovine serum albumin. Binding to salt-precipitated immunoglobulin of $N^{131}IP$–aminocap, the hapten of choice for this purpose, provides an assay for antibody measurable at concentrations down to at least M^{-9} serum binding capacity ($\sim 0{\cdot}1$ μg antibody/ml). Structurally related compounds and NIP–protein conjugates competitively inhibit binding of $N^{131}IP$–aminocap. The inhibitions indicate that the iodine contained in NIP, but not the carrier protein, contributes significantly to the binding site.

INTRODUCTION

A synthetic hapten, for which a simple assay of antibody based on direct binding could be designed, should prove useful for various purposes; we particularly hope to use such a system to investigate the nature of cellular recognition. In the search with this end, here described, we have been guided by the following considerations. The hapten should be immunologically powerful, i.e. a major fraction of the antibody elicited by hapten–protein conjugates should be directed against the hapten; it should be large enough for the protein carrier to play a minor role in binding to antibody; and the assay should depend on the binding of a radioactive hapten-containing compound to salt-precipitated antibody. The assay of choice is thus a development of the method introduced by Farr (1958) to measure direct binding of albumin antigens. This type of assay has a degree of sensitivity appropriate for survey of responsiveness in mice (Taylor, 1964; Mitchison, 1964).

The choice of assay in turn dictates certain features of the hapten. The only convenient isotope with which the requisite specific activity and ease of counting can be obtained is ^{131}I, and the iodine atom should itself contribute to the specificity of the determinant. In the assay the hapten not bound by antibody must remain in solution in concentrations of

salt high enough to precipitate immunoglobulins, i.e. at concentrations of ammonium sulphate over 35 per cent saturated, and preferably over 50 per cent saturated. The hapten must bind homogeneously, a condition which hapten conjugated to albumin will be here shown not to fulfil. In order to meet this condition the carrier must lack significant tertiary structure, as is presumably the case with polylysine, or should be dispensed with altogether. Finally, there are various minor requirements: the hapten must not bind to nonspecific proteins, and must be available at accurately known concentrations.

The properties of 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) were investigated towards this end. The compound should be antigenic by virtue of its nitro group, and would easily be labelled with isotopic iodine in the free *ortho* position to the phenolic group.

A variety of NIP-containing substances were synthesized and their suitability for measuring antibody-binding examined. After a satisfactory test had been found, based on binding of N^{131}IP–aminocaproic acid (N^{131}IP–aminocap), it was applied to a study of specificity and avidity of antibody. Alteration in carrier protein and hapten structure were then tested for their effect on inhibition of binding.

MATERIALS AND METHODS

Chemical preparations

A number of methods of coupling NIP and related compounds to the proteins were attempted. These include the use of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluenesulphonate, the preparation of mixed anhydrides and of activated nitrophenyl esters. No coupling was obtained by these methods.

The acid chlorides of 4-hydroxy-3-iodo-5-nitrobenzoic acid (NIB) and NIP could be coupled with proteins, but the yields were very low, owing to the rapid rate of hydrolysis of the acid chlorides. The azides of these compounds (cf. Gell, Harington and Pitt-Rivers, 1946) gave the best yields of coupled proteins.

4-*Hydroxy*-3-*nitrobenzoic acid* (*NB*). This compound is to be found in the literature (see Beilstein's *Handbuch der organischen Chemie*). A more simple preparation than those previously described is as follows: 4-hydroxybenzoic acid (34·5 g) was dissolved in acetic acid (250 ml) at 50–60°, and a solution containing nitric acid (20 ml, specific gravity 1·4) in acetic acid (75 ml), was added, with stirring, at such a rate that the temperature of the reaction mixture did not rise above 60°.

After the addition of the nitric acid solution was complete, the dark brown solution was evaporated under reduced pressure to about one-half of its original volume and the residual solution was kept at 10° for a few hours. The crystalline product was collected and washed with a little cold acetic acid. It was recrystallized from boiling water (about 500 ml). After drying *in vacuo*, the yield was 23 g of almost colourless crystals having melting point 184°.

4-*Hydroxy*-3-*iodo*-5-*nitrobenzoic acid* (*NIB*). This compound also appears in Beilstein's *Handbuch der organischen Chemie*, but the methods of preparation are laborious. A simple iodination of NB is as follows: 4-hydroxy-3-nitrobenzoic acid (7·32 g) was dissolved in acetic acid (250 ml) by warming at about 60°. To the stirred solution was added a solution of ICl (8 g) in acetic acid (50 ml). The temperature was maintained at 60° for 20 minutes. Water (500 ml) was then added and the product crystallized immediately. Excess iodine was removed by the addition of 10 per cent aqueous sodium metabisulphite solution. The product was collected and recrystallized from aqueous ethanol. The yield was 8 g, and the product had melting point 250°.

4-Hydroxy-3-iodo-5-nitrobenzoyl chloride (*NIB acid chloride*). This was prepared in the same way as NP acid chloride (see below). The product was not isolated; after removal of the thionyl chloride by repeated evaporation with benzene, the yield was assumed to be theoretical.

4-Hydroxy-3-iodo-5-nitrobenzoyl azide (*NIB azide*). The acid chloride from 1 m-mol NIB was dissolved in 3 ml dry acetone and cooled in an ice-bath. This solution was added to a chilled solution of 65 mg NaN_3 in 0·2 ml water, and the mixture was stirred for 20 minutes. After dilution with about 5 ml water the azide separated as an oil which crystallized on stirring. The yield was 70 per cent of the theoretical. The melting point was 94° (decomposition). (Found: C, 25·7; H, 1·3; N, 16·5 per cent. $C_7H_3O_4NI$ requires C, 25·2; H, 0·9; N, 16·8 per cent.)

4-Hydroxy-3-nitrophenylacetic acid (*NP*). 4-Hydroxy-phenylacetic acid (Koch, Light Laboratories Ltd) (10 g) was dissolved in glacial acetic acid (50 ml). The solution was cooled until the solvent just started to freeze (*c.* 12°). To the cooled solution was added a mixture containing concentrated nitric acid, specific gravity 1·4, (4 ml), and glacial acetic acid (8 ml). The addition was made at such a rate that the temperature remained below 15°. The dark brown coloured solution was left to stand at 15° for about 2 hours, after which time the bulk of the product had crystallized out. This was collected and recrystallized from boiling water. The yield was 7·5 g. The compound had a melting point of 145°. The molecular coefficient of extinction at pH 8·4 and 430 mμ was $4{\cdot}23 \times 10^3$. (Found: C, 48·8; H, 3·5; N, 7·2 per cent. $C_8H_7O_5N$ requires C, 48·9; H, 3·6; N, 7·1 per cent.)

4-Hydroxy-3-nitrophenacetyl chloride (*NP acid chloride*). 4-Hydroxy-3-nitrophenylacetic acid (NP) (0·5 g) was suspended in purified thionyl chloride (see below) (about 5 ml) and the mixture was refluxed in an apparatus protected with a calcium chloride tube. The flask was heated in a boiling water-bath. The solid quickly dissolved. After approximately 20 minutes, the thionyl chloride was evaporated under reduced pressure, leaving a deep yellow oil. Dry benzene (5 ml) was added, in which the oil was totally soluble, and the benzene was then evaporated under reduced pressure. This procedure was performed three times in all, and served to remove any residual thionyl chloride. The residual oil crystallized on cooling. After crystallization from dry benzene and ligroin the melting point was 100°. (Found: Cl, 16·3 per cent. $C_8H_6O_4NCl$ requires Cl, 16·5 per cent.)

Purification of thionyl chloride. The procedure is that given in *A Text-Book of Practical Organic Chemistry*, 2nd edn, by A. I. Vogel, p. 185, Longmans Green, London. *Note*—Some care must be exercised during the final stages of the distillation from linseed oil. When almost all of the thionyl chloride has distilled over, the distillation flask should be removed from the source of heat and cooled. If this is not done the remaining dark brown oil sometimes suddenly undergoes an exothermic reaction with the evolution of gas causing a thick tar-like foam to form, which is removed only with great difficulty from the apparatus.

4-Hydroxy-3-nitrophenacetyl azide (*NP azide*). NP acid chloride* (216 mg) (1 m-mol) was dissolved in 4 ml dry acetone and chilled in an ice-bath. The solution was added with stirring to a solution of 70 mg NaN_3 (British Drug Houses) in 0·25 ml water, also chilled. After a few moments, NaCl crystallized out. After 10 minutes 12 ml ice-cold water was added slowly to the reaction mixture. The NP azide separated as an oil which crystallized immediately. The product was collected, washed with water, and dried. The yield was about 80 per cent of the theoretical and the azide had a melting point of 61–62° (decomposition). (Found: N, 24·2 per cent. $C_8H_6O_4N_4$ requires N, 25·2 per cent.)

* The NP acid chloride was not usually weighed: the yield from 200 mg NP was assumed to be theoretical.

Note—All the azides in the hydroxyphenyl acetic acid series are unstable at room temperature and should be stored at −20°.

4-*Hydroxy*-3-*nitrophenacetyl*-ε-*amino-n-caproic acid* (*NP–aminocap*). NP azide (220 mg) (1 m-mol) was dissolved in 6 ml dry acetone and cooled in an ice-bath. ε-Amino-n-caproic acid (180 mg, 1·4 m-mol) was dissolved in 10 ml 0·24 M sodium bicarbonate. The two solutions were mixed and stirred for about 2 hours, the temperature of the cooling bath being allowed to rise to room temperature. The reaction mixture was kept at room temperature for at least 1 hour further and acidified with dilute HCl.

The crystalline NP–aminocap that separated was collected, washed with water and dried. The yield was about 80 per cent of the theoretical. The product had a melting point of 107–109°. (Found: C, 53·6; H, 5·6; N, 9·4 per cent. $C_{14}H_{18}O_6N_2$ requires C, 54·3; H, 5·8; N, 9·1 per cent.)

4-*Hydroxy*-3-*nitrophenacetylglycine* (*NP–gly*). The preparation was the same as for NP–aminocap. The yield was 83 per cent of the theoretical. The product had a melting point of 177–180° (decomposition). (Found: N, 11·4 per cent $C_{10}H_{10}O_6N_2$ requires N, 11·0 per cent.)

4-*Hydroxy*-3-*nitrophenacetylglycylglycine* (*NP–gly-gly*). The preparation was the same as for NP–aminocap. The yield was 87 per cent of the theoretical. The product had a melting point of 199° (decomposition). (Found: N, 13·8 per cent. $C_{12}H_{13}O_7N_3$ requires N, 13·5 per cent.)

α-N-*Acetyl*- ε-N-(4-*hydroxy*-3-*nitrophenacetyl*) *lysine* (*Ac-NP-lys*). NP azide (400 mg) in 8 ml acetone was added with stirring in the cold to a solution of α-*N*-acetyllysine (Neuberger and Sanger, 1943) in 18 ml 0·33 M bicarbonate. After 3 hours, about half the acetone was removed under diminished pressure and the solution was acidified with dilute HCl. The oily product which crystallized slowly was obtained in 70 per cent yield. It has a melting point of 155°. (Found: N, 11·3 per cent. $C_{16}H_{21}O_7N_3$ requires N, 11·4 per cent.)

4-*Hydroxy*-3-*nitrophenacetyl*-11-*aminohendecanoic acid* (*NP*–11). NP azide (222 mg) was dissolved in 6 ml dimethylformamide and added with stirring in the cold to a solution of 300 mg 11-aminohendecanoic acid in 12 ml water containing 0·6 ml triethylamine. After 16 hours at 2° the reaction mixture was filtered and acidified with dilute HCl. The product was recrystallized by dissolving in hot N NH_3 and acidifying with dilute HCl. The yield was 76 per cent of the theoretical. The compound had a melting point of 115–117°. (Found: C, 59·7; H, 7·2; N, 7·4 per cent. $C_{19}H_{28}O_6N_2$ requires C, 60·0; H, 7·4; N, 7·4 per cent.)

4-*Hydroxy*-3-*iodo*-5-*nitrophenylacetic acid* (*NIP*). NP (0·5 g) was dissolved in glacial acetic acid (25 ml). To the solution was added a solution containing ICl (0·5 g) in glacial acetic acid (5 ml). The mixture was heated at 60° for 30 minutes. Water (3 volumes) was added and the product began to crystallize. After cooling to room temperature excess iodine was removed by the addition of aqueous 20 per cent sodium metabisulphite solution. The crystals were collected and recrystallized by dissolving in boiling ethanol (10 ml) and adding water to the hot solution until crystallization just started. The yield after crystallization was 70 per cent of the theoretical. The product melted at 213° (decomposition). The molecular coefficient of extinction at pH 8·4 and 430 mμ was $4{\cdot}9 \times 10^3$. (Found: N, 4·2; I, 39·6 per cent. $C_8H_6O_5NI$ requires N, 4·3; I, 39·2 per cent.)

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetyl azide* (*NIP azide*). This preparation is best accomplished by the iodination of NP azide.

(1) NP azide (200 mg) was dissolved in glacial acetic acid (10 ml). ICl (160 mg) was

dissolved in glacial acetic acid (10 ml). The two solutions were mixed together and left to stand for 20 minutes at room temperature (20°). Ice-cold water (3 volumes) was then added slowly with stirring. The product was obtained as an oil which crystallized after the suspension had been left to stand in an ice-bath for about 20 minutes. The product was collected and dried *in vacuo*. The yield was 220 mg. The azide had a melting point of 65–66° (decomposition). (Found: N, 15·5 per cent. $C_8H_5O_4N_4I$ requires N, 16·1 per cent.)

(2) High specific activity N^{131}IP azide. Carrier-free Na^{131}I (200 μc in water—about 0·02 ml) was placed in a 10 ml stoppered conical test tube. An aqueous solution of ICl (see McFarlane, 1963) (0·031 ml) containing 0·41 mg I/ml was added to the Na^{131}I solution; the mixture was left at room temperature for 20 minutes for exchange to take place. A solution containing NP azide (500 μg/ml) in 0·2 M phosphate buffer, pH 6, was prepared, and 0·045 ml of this solution together with dimethyl formamide (0·1 ml) was added to the ^{131}ICl solution. The mixture was allowed to react at room temperature for 15 minutes and used directly for the reaction with the protein solution (see below).

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetyl*- ε-*amino-n-caproic acid* (*NIP–aminocap*). The preparation was the same as for NP–aminocap except that 1 m-mol of the NIP azide was dissolved in 8 ml acetone. The yield was about 50 per cent of the theoretical. The product melted at 152°. The molecular coefficient of extinction at pH 8·4 and 430 mμ was $5{\cdot}0 \times 10^3$. (Found: N, 6·1; I, 29·2 per cent. $C_{14}H_{17}O_6N_2I$ requires N, 6·4; I, 29·1 per cent.)

N^{131}*IP*- ε-*amino-n-caproic acid.* In this preparation the ICl was used in aqueous solution (see above). NP–aminocap was dissolved in water (1 mg/ml) and 0·01 ml of the solution was taken for the iodination. The reaction was carried out in a conical 15 ml glass-stoppered tube. The carrier-free Na^{131}I solution was placed in the bottom of the tube and equilibrated for 20 minutes at room temperature with 0·0125 ml of the aqueous ICl solution. The NP–aminocap solution (0·01 ml) and 0·2 M phosphate buffer, pH 6, (0·01 ml) were then added to the ICl solution and left to react at room temperature for 20 minutes. The product was separated from iodide on a column of Dowex resin AG.1×2 (chloride form: 200–400 mesh) manufactured by the Bio-Rad Laboratories.

The N^{131}IP–aminocap solution was transferred on to the resin column ($3 \times 0{\cdot}3$ cm) and the container was rinsed out three times with 0·25–0·3 ml water. The column was washed with a further 2×1 ml water and the N^{131}IP–aminocap was eluted with $3 \times 0{\cdot}5$ ml 50 per cent acetic acid. All the ^{131}iodide was retained on the column. The yield was about 70 per cent with respect to ^{131}I.

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetylglycine* (*NIP–gly*). NP–gly (100 mg) was dissolved in acetic acid (10 ml) and allowed to react at room temperature for 30 minutes with ICl (100 mg in acetic acid, 5 ml). The solution was concentrated to dryness under diminished pressure. The residue was recrystallized from boiling water. The yield was 103 mg and the product had a melting point of 168° (decomposition). (Found: N, 6·9; I, 32·5 per cent. $C_{10}H_9O_6N_2O$ requires N, 7·4; I, 33·5 per cent.)

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetylglycylglycine* (*NIP–gly-gly*). NP–gly-gly (100 mg) was iodinated as above with ICl (80 mg). The yield was 83 mg and had a melting point of 192° (decomposition). (Found: N, 9·1; I, 29·5 per cent. $C_{12}H_2O_7N_3I$ requires N, 9·6; I, 29·1 per cent.)

α-N-*acetyl*- ε-N-(4-*hydroxy*-3-*iodo*-5-*nitrophenacetyl*) *lysine* (*Ac-NIP-lys*). Ac-NP-lys (200 mg) was iodinated as above with 130 mg ICl. After removal of the acetic acid, the residue was crystallized by suspending in 10 ml hot water and adding ethanol till a clear solution

was obtained. The yield was 170 mg and the product had a melting point of 187–88°. (Found: N, 8·2; I, 26·0 per cent. $C_{16}H_{20}O_7N_3I$ requires N, 8·1; I, 25·8 per cent.)

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetyl*–11-*aminohendecanoic acid* (*NIP*–11). NP–11 (190 mg) was iodinated as above with ICl (90 mg). After 30 minutes, 2 volumes of water were added and the product collected after a further 30 minutes. It was recrystallized from ethanol (10 ml) by addition of water (6 ml) to the hot solution. The yield was 190 mg and the product melted between 125 and 130°. (Found: N, 5·4; I, 24·9 per cent. $C_{19}H_{27}O_6N_2I$ requires N, 5·5; I, 25·0 per cent.)

The preparation of N^{131}IP–11 was similar to that of N^{131}IP–aminocap.

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetyl*–11-*aminohendecanoylchloride*. This was prepared from 190 mg NIP–11 and 8 ml thionyl chloride. The oily product was deep yellow.

4-*Hydroxy*-3-*iodo*-5-*nitrophenacetyl*–11-*aminohendecanoicazide* (*NIP*–11 *azide*). The above acid chloride was dissolved in dry acetone (2 ml) and added with stirring in the cold to 30 mg NaH_3 in 0·2 ml water. After 20 minutes a brown oil was obtained on acidification which hardened at −20°. The product, which was very unstable, was used immediately for coupling with protein.

4-*Hydroxy*-3,5-*dinitrophenylacetic acid* (*NNP*). Prepared by the method of Wilkinson, (1956). The molecular coefficient of extinction of this compound at pH 8·4 and 445 mμ is $7{\cdot}12 \times 10^3$.

4-*Hydroxy*-3,5-*dinitrophenacetylchloride* (*NNP acid chloride*). NNP acid was converted to the acid chloride by the method used for NP acid chloride. The product was not isolated but was immediately converted to the azide.

4-*Hydroxy*-3,5-*dinitrophenacetylazide* (*NNP azide*). This compound was prepared from NNP acid chloride as for the preparation of NP azide. The compound was obtained in 90 per cent yield and had a melting point of 92° (decomposition). (Found: N, 25·7 per cent. $C_8H_5O_6N_5$ requires N, 26·2 per cent.)

4-*Hydroxy*-3,5-*dinitrophenacetyl*–ε-*amino-n-caproic acid* (*NNP–aminocap*). This compound was prepared from NNP azide by the method used for NP–aminocap. The yield was 66 per cent of the theoretical. The product had a melting point of 126–8°. (Found: N, 12·3 per cent. $C_{14}H_{17}O_8N_3$ requires N, 11·8 per cent.)

4-*Hydroxy*-3,5-*diiodophenylacetic acid* (*DIP*). Prepared according to Papa, Schwenk, Breiger and Peterson (1950). This compound has a molecular coefficient of extinction at pH 8·4 and 311 mμ $= 5{\cdot}64 \times 10^3$.

4-*Hydroxy*-3,5-*diiodophenacetylchloride* (*DIP acid chloride*). DIP was treated with thionyl chloride as in the preparation of NP acid chloride. The product was not isolated, but was immediately converted to the azide. (Found: Ag halide, 5·9 per cent. $C_8H_5O_2I_2Cl$ requires Ag halide, 6·1 per cent.)

4-*Hydroxy*-3,5-*diiodophenacetylazide* (*DIP azide*). The acid chloride from 500 mg of DIP was dissolved in 5 ml dry acetone and stirred in an ice-bath with 100 mg NaN_3 in 0·25 ml water. The azide started to crystallize immediately and was obtained in 89 per cent yield by further dilution with water. The product had a melting point of 73° (decomposition). No satisfactory analysis of this compound was obtained.

4-*Hydroxy*-3,5-*diiodophenacetyl*–ε-*amino-n-caproic acid* (*DIP–aminocap*). DIP azide (320 mg) was dissolved in 20 ml dry acetone and added with stirring to a solution of 134 mg ε-*amino-n-caproic* acid in 20 ml 0·2 M borate buffer, pH 8·4. After standing at room temperature for 16 hours some of the acetone was removed under diminished pressure. The product was crystallized twice from ethanol–water and once from acetic acid–water. The

yield was 120 mg. The product had a melting point of 143–5°. (Found: N, 2·8; I, 49·3 per cent. $C_{14}H_{17}O_4NI_2$ requires N, 2·7; I, 49·0 per cent.)

D^{131}IP–aminocap. This was prepared by exchange. DIP–aminocap (1 mg) was dissolved in 0·7 ml dimethylformamide and 0·3 ml 0·2 M phosphate buffer, pH 7·5. 0·01 ml of this solution+0·2 ml phosphate buffer, pH 7·5, was treated with 0·02 ml 0·001 N I_2 in KI and 200 μc ^{131}I, carrier-free. After standing at room temperature for 10 minutes one drop of 1 per cent Na metabisulphite was added and the product was separated from ^{131}iodide on a Dowex 1×2 column, as for N^{131}IP–aminocap. The yield of D^{131}IP–aminocap was 65 per cent with respect to ^{131}I.

Coupling of NIP azide with protein

Low coupling ratio 1–2 *NIP/HSA.* Human serum albumin (HSA) or bovine serum albumin (BSA) (1 g) was dissolved in 4 ml water plus 3 ml 1 M bicarbonate, and to it was added, with stirring, a solution of 8·6 mg NIP azide (1·6 mol/65,000) in 0·4 ml dimethylformamide. The reaction mixture was stirred for about 30 minutes and transferred to the refrigerator (2°) where it was kept overnight. The solution was then dialysed against several changes of 0·2 M bicarbonate or 0·2 M borate buffer, pH 8·4, until the diffusate was colourless.

In order to estimate the number of NIP or other hapten groups that were coupled to the proteins, the molecular coefficients of extinction of the haptens were determined in a Beckman Model D.U. spectrophotometer at λ_{max} and at pH 8·4. The values obtained are given in the experimental chemical section. Coupling ratio, 1·5 NIP/HSA. Coupling yield, 94 per cent.

High coupling ratio, about 10 *NIP/HSA.* HSA (or BSA) (10 g) was dissolved in 100 ml water plus 140 ml 0·2 M bicarbonate and to it was added, with stirring, a solution of NIP azide (860 mg) (16 mol/65,000) in 40 ml dimethylformamide. The solution was kept at 2° overnight. It was then dialysed against changes of 0·2 M bicarbonate or borate until the diffusate was colourless. Coupling ratio, 11·5–12·5 NIP/BSA. Coupling yield, 72–82 per cent.

High specific activity BSA. ^{131}I-labelled NIP azide from 22·2 μg NP azide (see above) was treated with a solution of 1 mg BSA in 0·4 ml 1 M bicarbonate buffer. The reaction mixture was kept at 2° for 16 hours. The solution was dialysed against several changes of 0·05 M borate buffer, pH 8·4, to remove labelled iodide and hydrolysed N^{131}IP azide. Yield with respect to radioactivity, and coupling ratio based on radioactivity, are given in Table 2.

Coupling of other haptens (e.g. DIP azide, NNP azide, etc.) was performed as above, according to the number of hapten groups per protein molecule required.

Proteins

Proteins and polyamino acids were obtained from the following sources: chicken serum globulin (CG) prepared by salt-fractionation (Eisen and Pressman, 1950); bovine serum albumin (BSA) from Armour, crystallized for ^{131}I-iodination, otherwise fraction V; human serum albumin (HSA), crystallized from Behringwerke ('reinst'); ovalbumin (OA), crystallized, from Worthington; gelatine from British Drug Houses; poly-L-lysine HBr from Yeda; edestin, crystallized, from Nutritional Biochemicals.

Antisera

Antisera against NIP conjugates (10–15 groups/mol) were prepared in rabbits by immunization with alum-precipitated antigen, or with Freund's adjuvant according to the procedure of Porter (1955). Antisera against similar conjugates were prepared in

mice by immunization with alum-precipitated antigen mixed with pertussis (Brownstone, Mitchison and Pitt-Rivers, 1966).

Serological

In binding tests the antiserum was initially diluted 1:10 in pH 8·4 borate buffer (Farr, 1958), and then further dilutions were made into 10 per cent normal rabbit serum in borate buffer. The hapten or antigen was then added in an equal volume to yield a final concentration of M^{-8} (hapten) or 1 μg/ml (antigen). After incubation overnight at 4° an equal volume of saturated ammonium sulphate was addded. The tubes were then shaken and centrifuged; after discarding the supernatant, the radioactivity in the precipitates was measured without washing in a well-crystal scintillation counter. The binding is expressed as a percentage of the total radioactivity added to each tube, after subtraction of the background radioactivity detected in tubes containing hapten or antigen plus 10 per cent normal rabbit serum.

Precipitin tests were carried out and analysed by the method of Eisen, Carstein and Belman (1954), using the optical density 280 mμ to estimate total protein and the peak absorption of the hapten to estimate antigen. Binding capacities for albumin antigens were measured by a simplified version of the Farr method (Mitchison, 1964).

Inhibition tests were carried out by preparing a suitable series of ten two-fold dilutions of inhibitor. To 0·05 ml samples of these were added 0·2 ml of the radioactive hapten ($2 \times M^{-8}$). Next, 0·25 ml of antibody was added, diluted in 10 per cent normal rabbit serum to yield 50–70 per cent binding in the inhibitor-free controls. After incubation, 0·5 ml saturated ammonium sulphate was added and the tubes shaken and centrifuged. The supernatants were then discarded and the radioactivity of the precipitates measured.

RESULTS

ANTISERA

An analysis of the principal antisera used in the binding tests is given in Table 1. Both antisera were raised in rabbits, the NIP–CG antiserum with Freund's adjuvant, and the NIP–OA antiserum without. Both are pools of three to five individual sera, and in no case did an individual rabbit fail to respond.

TABLE 1

PRECIPITIN VALUES FOR RABBIT ANTISERUM TO NIP CONJUGATES

Immunization antigen	Precipitin antigen	Antibody (mg/ml)	Molar conc. of anti-NIP antibody* ($\times M^{-8}$)	$N^{131}IP$–aminocap molar binding capacity ($\times M^{-8}$) at M^{-8} hapten
NIP–CG (12 hapten/mol)	NIP–CG (12 hapten/mol)	6·35	—	—
	NIP–BSA (11 hapten/mol)	0·72	450	690
NIP–OA (15 hapten/mol)	NIP–OA (15 hapten/mol)	0·36	—	—
	NIP–BSA (11 hapten/mol)	0·19	119	48

* Assuming that: (i) antibody molecular weight = 160,000; and (ii) antibody precipitated by NIP–BSA is total anti-NIP.

CHOICE OF HAPTEN OR ANTIGEN FOR USE IN BINDING TEST

Various materials which might have proved suitable for use in the binding test were prepared, as listed in Table 2. The aim in every case was to obtain high specific radioactivity, in order to be able to dilute the material to at least M^{-8} and so obtain high sensitivity in the test. In the case of $N^{131}IP$–BSA and $N^{131}IP$–gelatine the radioactive azide was first prepared, and then reacted with the protein; this is not an entirely satisfactory procedure, since high specific activity material is involved in two steps with consequent hazards and risk of loss. With $N^{131}IP$–polylysine and $N^{131}IP$–aminocap, on the

TABLE 2

SUBSTANCES PREPARED FOR USE IN BINDING TEST

	NIP/mol	Molecular weight	Efficiency of radio-iodination* (%)	Solubility in 50 per cent saturated ammonium sulphate (%)	Binding by NIP–CG antiserum†
$N^{131}IP$–BSA	6·5	70,000	27	95	Heterogeneous
$N^{131}IP$–polylysine A	5·2	42,000‡	10–20	93	Homogeneous
$N^{131}IP$–polylysine B	1·5–10	42,000	10–20	0–32	Homogeneous
$N^{131}IP$–polylysine	3	17,000	12	78	Homogeneous
$N^{131}IP$–gelatine	23	110,000	—	<5	—
$N^{131}IP$–aminocap	1	436	60–70	100	Homogeneous

* With 0·1–0·01 μmoles material.
† As judged from binding curve, see text.
‡ Correct value may be less, see text.

other hand, the non-iodinated precursor was synthesized in bulk, and radio-iodination then carried out in a single step. The same procedure might have been applied to $N^{131}IP$–gelatine, although in this case some iodination would have occurred outside the NIP determinant in the few tyrosyl side-chains of gelatine.

The $N^{131}IP$–gelatine proved insoluble in 50 per cent saturated ammonium sulphate and was not further investigated. Several bulk preparations (~ 100 mg) of NP–polylysine were made from a single batch of polylysine HBr (molecular weight 41,000). The iodinated product from most of these proved somewhat insoluble in 35–50 per cent saturated ammonium sulphate, even after centrifugation (100,000 ***g***, 30 minutes) to remove aggregated material. Exceptional results were obtained with one bulk preparation of NP–polylysine ('A' in Table 2); radio-iodination on repeated occasions invariably yielded an adequately soluble product, of which 95 per cent of the radioactivity proved capable of binding to anti-NIP antibody. The lower molecular weight batch of polylysine HBr (molecular weight 17,000) yielded a $N^{131}IP$-conjugate that was less erratically soluble in 50 per cent saturated ammonium sulphate; we conclude that one or more peptide bonds split during the synthesis of preparation 'A'.

Both $N^{131}IP$–BSA and $N^{131}IP$–aminocap proved adequately soluble in 50 per cent saturated ammonium sulphate, but the two materials differed markedly in their binding properties. These are illustrated in Figs. 1 and 2. Binding of $N^{131}IP$–polylysine and $N^{131}I$-P–aminocap by NIP antibody, and $N^{131}IP$–BSA by BSA antibody, evidently follow similar curves. In fitting a standard curve to these observations we assume that binding is

proportional to antibody concentration at less than 35 per cent binding, and that at over 35 per cent binding it is proportional to log (antibody concentration); in other words, the calculated binding capacity of an antiserum is independent of the concentration at which it is measured, provided that binding is less than 35 per cent. The curves shown in the figures, which are drawn on these assumptions through the mean of the observed values, give a good fit. Our belief in the validity of these assumptions is reinforced by extensive tests with early and late antisera made at six-fold dilutions—our standard test in subsequent work—where good agreement has been obtained in the binding capacity calculated from tubes containing different concentrations of antiserum. Furthermore, the same binding curve fits data obtained with BSA and other albumins (Mitchison, 1964).

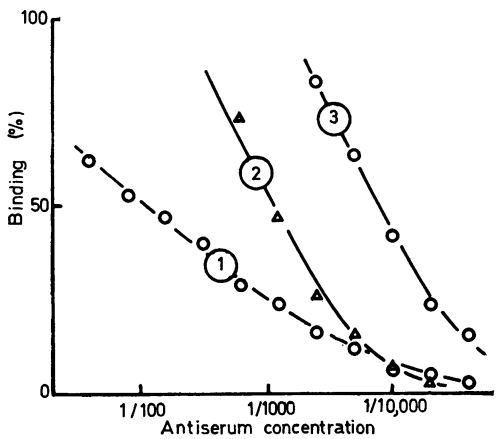

FIG. 1

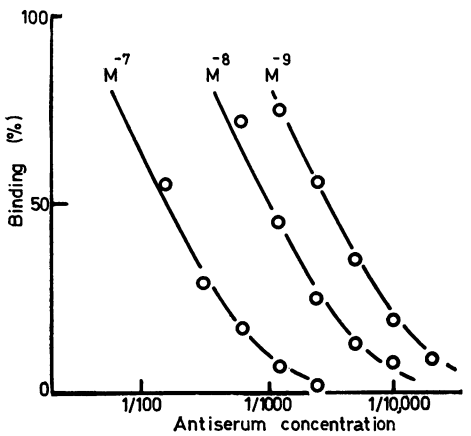

FIG. 2

FIG. 1. Binding as a function of antiserum concentration. (1) $N^{131}IP$–BSA bound by NIP–CG antiserum; (2) $N^{131}IP$–PL bound by same NIP–CG antiserum; and (3) same $N^{131}IP$–BSA bound by BSA antiserum. Curve (1) fitted by eye; curves (2) and (3) fitted by method described in text.

FIG. 2. Binding of $N^{131}IP$–aminocap as a function of NIP–CG antiserum concentration; $N^{131}IP$–aminocap at M^{-7}, M^{-8}, M^{-9} total concentration.

Binding of $N^{131}IP$–BSA by NIP antibody is by this standard anomalous. A relatively flat curve is obtained (Fig. 1) due presumably to heterogeneity of binding sites on the antigen. Some of the $N^{131}IP$ sites appear to be relatively inaccessible, while others, to judge from convergence of the two curves, are as accessible as the $N^{131}IP$ sites on polylysine. For this reason the use of $N^{131}IP$–BSA was not pursued, and further work was carried out with $N^{131}IP$–aminocap and other equivalent haptens.

We shall express the *molar binding capacity* of antisera as their capacity to bind in the range below 35 per cent binding.

OTHER ^{131}I-CONTAINING HAPTENS

$N^{131}IP$–aminocap is identical with the terminal portion of the group produced by linkage of NIP to a free lysine ε-amino group, and thus resembles the determinants of the conjugates used for immunization. Binding of the hapten to antibody might, however, be further improved by inclusion of a second amine in the α position, or by linking a dipeptide to NIP. The compounds $N^{131}IP$–lys, $N^{131}IP$–gly, and $N^{131}IP$–gly-gly were therefore synthesized, and their binding compared with that of $N^{131}IP$–aminocap. The binding of all three new compounds followed the standard curve (<35 per cent

linear, > 35 per cent logarithmic), and so valid comparisons could be made. At M^{-8} hapten concentration these three haptens and $N^{131}IP$–aminocap were all bound to approximately the same extent by antiserum against NIP-conjugate (but see Fig. 3).

In order to test whether the nitro group plays an important part in determining immunizing power, DIP conjugates were tried out. Their antisera were assayed with $D^{131}IP$–aminocap, a hapten which again displayed the standard binding curve. Cross-reactions with NIP antisera were not tested, except by the inhibition method described below.

With the aim of testing, in the future, the consequences of separating NIP from the carrier protein by a bridge, immunizations were performed with NIP–11 conjugates. Some antisera were assayed with $N^{131}IP$–11, a hapten which again displayed standard binding; for comparison, $N^{131}IP$–aminocap binding was tested. In general $N^{131}IP$–

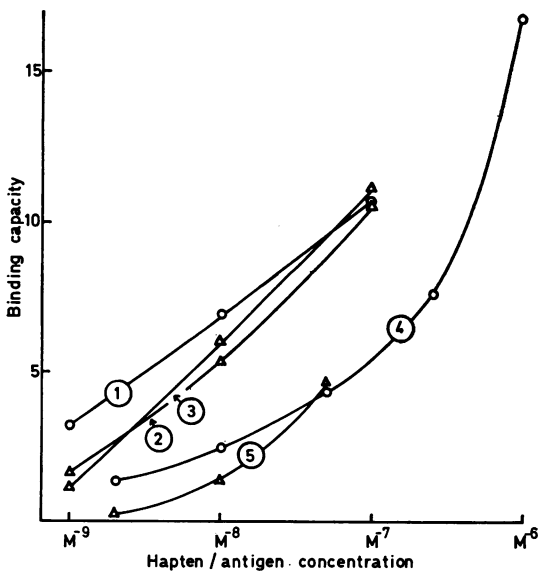

FIG. 3. Binding capacity as a function of hapten/antigen concentration. (1) $N^{131}IP$–aminocap, (2) $N^{131}IP$–gly, and (3) $N^{131}IP$–gly-gly bound by same NIP–CG antiserum; binding capacity $\times M^{-8}$; (4) ^{131}I–HSA, binding capacity $\times M^{-8}$, and (5) ^{131}I–BSA, binding capacity $\times M^{-7}$ (assume molecular weight 70,000 for HSA, BSA); bound by same HSA antiserum.

aminocap was bound in larger amount than $N^{131}IP$–11. For example, an antiserum against NIP–11–CG had a binding capacity of $32 \times M^{-8}$ for $N^{131}IP$–aminocap and only $20 \times M^{-8}$ for $N^{131}IP$–11 (62 per cent); and an antiserum against NIP–CG had a binding capacity of 160 for $N^{131}IP$–aminocap and only 72 for $N^{131}IP$–11 (45 per cent). Antibody specific to NIP–11 could be detected only by the inhibition method described below.

DILUTION EFFECT

Lowering the concentration of hapten is likely to improve the sensitivity of the binding test; and at low concentrations binding is likely to become more selective. Hitherto binding has been measurable at concentrations down to M^{-8} (Dubert, 1959; Nissonoff and Pressman, 1959). By the present method binding can be measured at hapten concentrations down to at least M^{-9}. The use of 100 μc ^{131}I for iodination yields a product which gives ~ 1000 counts/min at M^{-9}. Throughout the range tested (M^{-7} to M^{-9}) the standard binding curve was obtained (Fig. 2).

The binding capacity decreases when an albumin antigen is diluted (Farr, 1958; Mitchison, 1964), to an extent which varies from one antiserum to another. The dilution effects obtained with N^{131}IP–aminocap have not been systematically investigated, but it is evident from the data shown in Fig. 3, obtained with comparable antisera, that hapten and protein dilution effects—and therefore presumably association constants—are quantitatively similar.

Cross-reactive haptens, like cross-reactive proteins, can be expected to display relatively less binding at lower concentrations (Dubert, 1959). Fig. 3 shows the binding of ^{131}I–BSA by the HSA antiserum, and of N^{131}IP–gly and N^{131}IP–gly-gly by the NIP antiserum. The relative binding decreases with dilution in each case (the overlap of the NIP binding curves at high hapten concentration falls within the limits of experimental error). According to this criterion, the reactions with the NIP–glycine peptides constitute cross-reactions.

INHIBITION

A further guarantee of the specificity of binding of the radioactive haptens, and a general method for testing binding of non-radioactive substances, is provided by inhibition. Inhibition of binding resembles in principle inhibition of precipitation, but can be carried out at lower concentrations and therefore permits examination of poor inhibitors.

Typical curves of inhibition are shown in Fig. 4. We employ the method of analysis of Pauling, Pressman and Grossberg (1944) to evaluate for each inhibitor two constants: the average bond-strength relative to a reference hapten (inhibition constant) K'_0 and a heterogeneity index σ. The reference hapten is normally the same as the radioactive hapten but in the form of a non-radioactive solution made up from a bulk preparation. Although

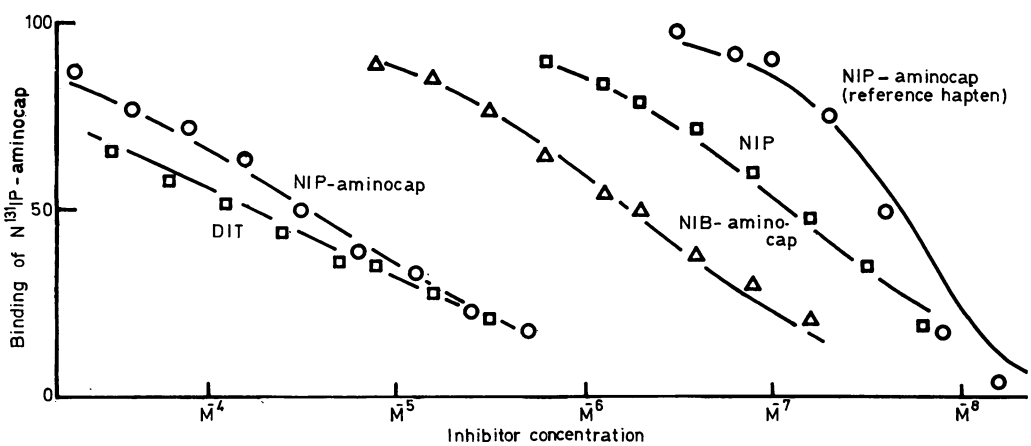

FIG. 4. Binding of N^{131}IP–aminocap as a function of inhibitor concentration. Abbreviations for inhibition as in Table 3 and text. N^{131}IP–aminocap M^{-8}; NIP–CG antiserum diluted to yield 60 per cent binding without inhibition.

the theory was developed originally for inhibition of precipitation, it fits the present data adequately, as can be seen in Fig. 4. Results obtained with a series of inhibitors are shown in Table 3. The stronger inhibitors give K'_0 and σ values in the range described by Pauling *et al.*, but the present series includes structurally more remote haptens than have been tested previously. A wide inhibition spectrum of this kind can be obtained without using

much antibody. Approximately M^{-11} moles of antigen binding capacity (~ 1 μg antibody) is required for analysis of each inhibitor and the quantity can be reduced still further by working at a lower binding value.

TABLE 3

INHIBITION OF N^{131}IP–AMINOCAP BINDING TO NIP–CG ANTISERUM BY STRUCTURALLY RELATED HAPTENS

	K'_0	σ
NIP–aminocap (4-Hydroxy-3-iodo-5-nitrophenacetyl–ε-amino-n-caproic acid)	1	<1
NIP (4-Hydroxy-3-iodo-5-nitrophenylacetic acid)	0·25	2–3
NNP–aminocap (4-Hydroxy-3,5-dinitrophenacetyl–ε-amino-n-caproic acid)	0·11	3–4
NIB–aminocap (4-Hydroxy-3-iodo-5-nitrobenzoyl–ε-amino-n-caproic acid)	0·046	3
NIB (4-Hydroxy-3-iodo-5-nitrobenzoic acid)	0·011	5
NNP (4-Hydroxy-3,5-dinitrophenylacetic acid)	0·0078	4
NP–aminocap (4-Hydroxy-3-nitrophenacetyl–ε-amino-n-caproic acid)	0·00085	4
DIT (3,5-Diiodo-L-tyrosine)	0·00046	5
NP (4-Hydroxy-3-nitrophenylacetic acid)	0·000053	5
MIT (3-Iodo-L-tyrosine)	0·0000063	5

A qualitative understanding of the binding site can be gained from these experiments. The presence of nitro-, iodo- and methylene groups contribute in decreasing order to the strength of binding; the amide bond and lysine side chain contribute as well, but to a lesser extent. A second nitro-group can substitute partially for the iodine. NIP conjugates, then, provide the first example of an antigen in which iodine plays a well-defined role in a determinant group, a matter which may prove useful for *in vivo* work with labelled antigen.

No account has been taken in these experiments of non-specific binding of the haptens by serum proteins. Since N^{131}IP–aminocap is not itself bound to any detectable extent, non-specific binding of structurally related compounds is unlikely to be important. Furthermore the concentration of serum protein is kept constant throughout the inhibition range.

The binding of structurally similar haptens can be distinguished more accurately by inhibition than by direct measurement. Provided that both are available at high specific radioactivity, each can be used to inhibit binding of the other by the same antiserum. Furthermore the inhibition data are more trustworthy because the test compound can be weighed out directly. The results obtained by application of the inhibition test to a series of radioactive haptens is shown in Table 4. From these tests NIP–aminocap emerges equal in bond strength to NIP–lys; both are bound more strongly than their related haptens. As might be expected, NIP–11 is bound more strongly to antiserum against its own conjugate.

TABLE 4

INHIBITION CONSTANTS (K'_0) OBTAINED WITH NIP–CG ANTISERUM PREPARED IN RABBITS (EXCEPT FOR * OBTAINED WITH NIP–11–CG ANTISERUM PREPARED IN MICE)

Inhibitor	Radioactive hapten				
	N^{131}IP–aminocap	N^{131}IP–gly-gly	N^{131}IP–gly	N^{131}IP–lys	N^{131}IP–11
NIP–aminocap	1·0	1·8	1·6	1·0	2·8 (0·27)*
NIP–gly-gly	0·42	1·0	1·1	—	—
NIP–gly	0·22	0·83	1·0	—	—
NIP–lys	1·0	—	—	1·0	—
NIP–11	0·14	—	—	—	1·0

We have also used inhibition to test for carrier-specificity in NIP antisera. The antisera, inhibitors, and the results obtained are shown in Table 5. The antisera were all obtained at least 6 weeks after immunization with the conjugate in adjuvant. The activity of the inhibitory conjugates has been calculated per mole of hapten. No significant carrier-specificity was detected by this test; nor have similar tests with early sera (6–9 days post-immunization) been any more successful in detecting carrier specificity. On the other hand, reducing the coupling ratio markedly reduced inhibitory power. This may be due to the fact that the first NIP groups to couple are inaccessible.

TABLE 5

INHIBITION CONSTANTS (K'_0) OF NIP-CONJUGATES: LACK OF CARRIER SPECIFICITY

		Antisera		
		Rabbit anti-NIP–CG	Rabbit anti-NIP–OA	Mouse anti-NIP–CG
NIP–CG	(11 hapten/mol)	0·60	0·55	0·44
NIP–OA	(15 hapten/mol)	0·52	0·69	0·28
NIP–BSA	(11 hapten/mol)	0·52	0·51	0·56
NIP–BSA	(1·5 hapten/mol)	—	0·098	0·028
CG		$<0·0001$*	—	—

* i.e. no inhibition detected in presence of CG (1000 μg/ml).

DISCUSSION

Binding of $N^{131}IP$–aminocap by salt-precipitated antibody emerges from these tests as a potentially useful assay. It reads in a range (M^{-6} to M^{-9} binding capacity) which conveniently spans the normal primary and secondary responses of mice (Mitchison, 1964; Brownstone *et al.*, 1966), and it is backed up by a much more sensitive bacteriophage-neutralization method (Mäkelä, 1966). This assay avoids some of the difficulties associated with other types of hapten-specific assay: no carrier protein is involved, nor is antibody needed in large amounts or in a highly purified condition.

In principle the assay is similar to the direct binding assay for antibody to albumins (Farr, 1958), and it is open to the same doubts. The most serious question concerns the binding curve, which in neither case has yet been properly explained: why does the relationship change at 35 per cent binding, for example? The mere fact that the same binding curve has been obtained with several sera and at several concentrations of hapten justifies the assay empirically, but we can go further than this without a full theory. The 'standard' binding curve (<35 per cent linear; $>$ 35 per cent logarithmic) has now been obtained with $N^{131}IP$–aminocap, several other $N^{131}IP$–containing monovalent haptens, $N^{131}IP$–polylysine, and several crystalline albumins (bovine, human and egg); it can also be obtained with highly purified muramidase and diphtheria toxoid (unpublished data). On the other hand, relatively flat binding curves are obtained with less homogeneous antigens: $N^{131}IP$–BSA in the present experiments, and a *Vibrio cholerae* antigen (Freter, 1962). It is therefore likely that (a) the 'standard' curve is the steepest obtainable, and is the mark of a homogeneous antigen, and (b) the change in the 'standard' curve above 35 per cent binding is not due to formation of antigen–antibody complexes.

The association constant can be estimated from the antigen dilution effect, but this is not formally acceptable because salt precipitation may not accurately 'freeze' the equilibrium. The relationship of binding capacity to precipitin content appears to be variable (Table 1), as might be expected; binding of hapten is presumably less likely to be effected by antibody with partial carrier-specificity, and variation in association constant openly influences binding.

N^{131}IP–aminocap is the hapten of choice for the binding test. The precursor NP–aminocap can be conveniently iodinated at high specific radioactivity, and is itself more easily prepared than the alternative amino acid derivatives. From the binding, and particularly from the inhibition, results it seems unlikely that much would be gained by the addition of further structure at the 'carrier' end.

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