Interaction of Dinitrophenyl Groups Bound to Bovine Serum Albumin with Univalent Fragments of Anti-Dinitrophenyl Antibody

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Two lysine residues of bovine serum albumin reacted with 1-fluoro-2,4-dinitrobenzene with apparent second-order rate constants approx. 500-times greater than those observed in similar reactions with low-molecular-weight lysine derivatives. A series of dinitrophenyl (Dnp)-bovine serum albumins were prepared and their ability to bind univalent fragments of anti-Dnp antibody was measured by fluorescence-quenching titrations. Compared with the Dnp group of the free hapten, 6-*N*-Dnp-aminohexanoate, the majority of the protein-bound Dnp groups were unavailable to the antibody at pH 8.0. When the same Dnp-albumins were titrated at pH 3.0 the availability of the Dnp groups increased approx. 3-fold. Dnp-albumins were treated with pepsin at pH 3.0 and Dnp-containing fragments isolated by chromatography on DE-52 DEAE-cellulose. Fluorescence-quenching titrations showed that the Dnp groups on the fragments behaved like the free hapten with respect to quenching efficiency, although with an increased dissociation constant. The association between the Dnp-albumins and the antibody was measured also by difference-spectral titrations at high protein concentrations. Antibody binding was increased under these conditions, but the Dnp group of mono-Dnp-albumin remained unavailable to antibody. We propose that the reactive lysine residues are located in clefts between the globular sub-domains of the single polypeptide chain. Dnp groups attached to these lysine residues are fully exposed to the solvent, but binding of the macromolecular probe, anti-Dnp antibody, is sterically hindered by the adjacent surface of the albumin molecule.

We have described previously (Knight & Green, 1976) a new technique for probing the environment of the amino acid residues in proteins that can be labelled with Dnp groups. Fluorescence-quenching titrations showed that Dnp groups attached by aminoalkylsuccinimide chains to the thiol group of bovine mercaptalbumin were fully available to univalent fragments of high-affinity antibody to Dnp, even when the alkyl chain was short. Similar results were given by mono-Dnp-ribonuclease A carrying the Dnp group on the lysine-41 residue. The apparent dissociation constants of the protein-bound Dnp groups were, however, significantly greater than that of the interaction between anti-Dnp antibody and the free hapten, 6-*N*-Dnp-aminohexan-1-oate. In the present paper we describe the use of univalent fragments of high-affinity antibody to Dnp in a study of the environment of the *N*-Dnp-lysine groups in *N*-Dnp-bovine serum albumin.

Bovine serum albumin contains two lysine residues that react rapidly with 1-fluoro-2,4-dinitrobenzene (Green, 1963). The rate of this reaction is considerably decreased by low concentrations of non-

polar anions, and shows saturation kinetics when the concentration of 1-fluoro-2,4-dinitrobenzene is increased. Green (1963) proposed that the reactive lysine residues were located adjacent to a non-polar binding site for 1-fluoro-2,4-dinitrobenzene. A kinetic study of the reaction of the lysine residues of human serum albumin with trinitrobenzenesulphonic acid (Goldfarb, 1966) indicated that they were also grouped in subsets each of which was described by an average velocity constant. Moreover, the number in each subset was analogous to the number of non-polar anion-binding sites in the different classes described by Goodman (1958) and Fletcher *et al.* (1970). In serum albumin these binding sites are thought to be located in clefts in the protein surface that mark the interfaces between separate globular portions of the single polypeptide chain (Foster, 1960). This model would suggest that Dnp groups bound to the most reactive lysine residues may lie within the van der Waals' surface of the protein. Such Dnp groups would be unable to bind anti-Dnp antibody (Knight & Green, 1976). On the other hand it has been suggested that the more reactive lysine residues are more exposed to solvent (Goldfarb, 1966) and on this basis the Dnp groups should be available to antibody.

Abbreviation used: Dnp, dinitrophenyl.

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We report here the results of a more detailed study of the kinetics of the reaction between bovine serum albumin and 1-fluoro-2,4-dinitrobenzene. *N*-Dnp-bovine serum albumins have been made with increasing numbers of Dnp groups/molecule of protein and the interaction of these Dnp-proteins with univalent fragments of anti-Dnp antibody has been studied by fluorescence-quenching titrations (Velick *et al.*, 1960) and by spectrophotometric titrations (Little & Eisen, 1967).

At pH values below 4 bovine serum albumin undergoes a structural transition accompanied by an increased exposure of hydrophobic regions of the molecule (Foster, 1960). Digestion of the unfolded protein by pepsin at pH 3 releases globular fragments (Weber & Young, 1964). We have therefore studied the effect of acid pH values and pepsin digestion on the accessibility of albumin-bound Dnp groups to univalent fragments of anti-Dnp antibody.

Materials and Methods

Bovine serum albumin (crystalline, salt-free) was obtained from Armour Pharmaceutical Co., Eastbourne, East Sussex, U.K. 1-Fluoro-2,4-dinitrobenzene was supplied by BDH Chemicals, Poole, Dorset, U.K. 6-*N*-Dnp-aminohexanoic acid was synthesized by the method of Carsten & Eisen (1953). 6-*N*-Dnp-lysine hydantoin (Ettinger & Hirs, 1968) was synthesized by the method of Stark & Smyth (1963). Other reagents were obtained commercially and were of the highest purity available.

Preparation of *N*-Dnp-bovine serum albumin

Bovine serum albumin (Armour, lot MH 2470) had a fatty acid content of 0.8 mol/mol determined by the method of Chen (1967). Before the reaction with 1-fluoro-2,4-dinitrobenzene the single reactive tyrosine (Shaw, 1962) and cysteine (Hughes, 1947) residues were blocked with di-isopropyl phosphorofluoridate and iodoacetamide respectively. Bovine serum albumin (7.5 μ mol) was dissolved in 0.1 M-sodium phosphate, pH 8.0 (25 ml). Di-isopropyl phosphorofluoridate (0.1 mmol) in propan-2-ol (0.1 ml) was added and the solution was stirred overnight at 4°C. Iodoacetamide (75 μ mol) was added with stirring at 21°C. After 20 min, 2-mercaptoethylamine hydrochloride (0.35 mmol) was added and the solution stirred for 30 min, then 2 M-HCl was added to adjust the pH to 5.0 and the solution passed through a column (1.5 cm $\times$ 11 cm) of Dowex 50 (X8; Na⁺ form). Fractions (4 ml) with an A_{278} greater than 2.0 were combined (32 ml) and dialysed against 0.05 M-sodium borate, pH 9.0, containing 0.05 M-NaCl (2 $\times$ 1000 ml) at 4°C. After dialysis the concentration of albumin

was measured spectrophotometrically, assuming an $A_{1\text{cm}}^{1\%}$ of 6.8 at 278 nm (Tombs *et al.*, 1959) and a mol.wt. of 66 500 (Baldwin, 1957). The thiol content, determined by the method of Ellman (1959), was less than 0.03 mol of thiol/mol. The thiol group could also be blocked by addition of a 3-fold excess of *N*-ethylmaleimide.

N-Dnp-albumins were prepared from portions of this stock solution (approx. 0.25 mm). 1-Fluoro-2,4-dinitrobenzene (50 mm) in ethanol was added with stirring and the solution was left in darkness at 21°C overnight. To obtain up to 5 mol of *N*-Dnp groups/mol of albumin it was necessary to add 1.5 times the equivalent amount of 1-fluoro-2,4-dinitrobenzene. The reaction at lysine became less efficient at higher degrees of substitution and it was necessary to add 3 times the equivalent amount of reagent to obtain 30 or more mol of *N*-Dnp groups/mol of albumin. Dinitrophenol was removed by passing the solution through a column (0.4 cm $\times$ 6 cm) of Dowex 2 (X8; Cl⁻ form) eluted with 0.2 M-sodium phosphate, pH 8.0. Eluent containing the yellow Dnp-protein was collected and the volume measured. The absorption spectrum was recorded. The concentration of *N*-Dnp groups was calculated by assuming $\epsilon = 17\,500 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 363 nm (Eisen & Siskind, 1964). The protein concentration was calculated from the degree of dilution of the original stock solution. Dnp-albumin solutions were stored frozen at -20°C.

The absorption spectra of Dnp-labelled albumins showed A_{305}/A_{363} ratios greater than those observed with *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins (Knight & Green, 1976), suggesting the presence of *O*-Dnp-tyrosine. The content of this derivative was calculated as follows. The *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumin spectra were used to calculate the protein contribution to the absorbance at 305 nm ($\epsilon = 3200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). The *O*-Dnp-tyrosine contribution was calculated on the assumption that the remaining absorbance at 305 nm was due only to it and to *N*-Dnp-lysine, whose contribution was taken as $0.12 \times A_{363}$ [from the absorption spectrum of 6-*N*-Dnp-lysine hydantoin (Ettinger & Hirs, 1968)]. The absorption coefficient of *O*-Dnp-tyrosine is $11\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 305 nm (Sanger, 1949).

Reaction kinetics of bovine serum albumin with 1-fluoro-2,4-dinitrobenzene

The protein solution (12 μ M) in 0.1 M-sodium borate buffer, pH 8.5 (10.5 ml), at 25°C was mixed with a portion (0.5 ml) of a solution of 1-fluoro-2,4-dinitrobenzene (0.2 M, in ethanol). At intervals portions (0.5 ml) of the reaction mixture were removed and added to 0.14 M-acetic acid (0.5 ml) to give a final pH value of approx. 5.8. Dinitrophenol was removed by passing the solution through a column (0.3 cm $\times$ 5 cm;

0.35 ml) of Dowex 2 (X8; Cl^- form). The A_{400} of the eluent was measured in cuvettes of 1.0 cm path length. The reference cuvette contained 1-fluoro-2,4-dinitrobenzene (4.6 mm) in the borate buffer/acetic acid mixture.

The removal of dinitrophenol was unnecessary during the rapid initial stage of the reaction, which therefore was monitored continuously at 400 nm in a recording spectrophotometer (Unicam SP.700) with 0.5 cm-path-length cuvettes. The concentrations of the reactants were identical with those used in the sampled runs. The reference cuvette contained 1-fluoro-2,4-dinitrobenzene (9.2 mm) in 0.1 M-sodium borate buffer, pH 8.5. The progress curves of the reaction showed an initial exponential increase in absorbance superimposed on a linear component corresponding to the modification of the least reactive lysine residues. The exponential part of the reaction course was corrected for this slow component (cf. Gerig & Reinheimer, 1975). The corrected progress curves were analysed by assuming that the reactive lysine residues were grouped into subsets with different pseudo-first-order rate constants. The methods of analysis have been described by Goldfarb (1966) and Freedman & Radda (1968). The concentration of *N*-Dnp groups was calculated assuming $\epsilon = 9000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 400 nm (Eisen & Siskind, 1964; Ettinger & Hirs, 1968).

Pepsin digestion of Dnp-labelled albumin

The conditions for pepsin digestion were determined by using the unlabelled protein (Weber & Young, 1964). A solution of bovine serum albumin (2 ml, 25 mg/ml) was adjusted to pH 3.0 by the addition of 1 M-HCl, and buffered with 5 mM-sodium citrate/phosphate buffer, pH 3.0. Pepsin (50 μg) was added and the mixture incubated at 25°C. At intervals portions (50 μl) were withdrawn and diluted into a solution of 8-anilinonaphthalene-1-sulphonic acid (0.2 mm) in 0.1 M-sodium phosphate, pH 7.0 (2 ml). The fluorescence at 470 nm was measured. Digestion was continued for 70 min, at which time the fluorescence efficiency had fallen to 25% of the initial value (Weber & Young, 1964). Pepsin digestion was stopped by the addition of 1 M- K_2HPO_4 (0.1 ml) and the digest was dialysed against 0.02 M-sodium phosphate buffer, pH 6.0 (2 $\times$ 100 ml). The dialysed solution was applied to a column (0.8 cm $\times$ 11 cm) of Whatman DE-52 DEAE-cellulose. The column was eluted at 4°C with a linear gradient from 0.2 M-sodium phosphate, pH 6.0 (50 ml), to 0.33 M-sodium phosphate, pH 6.0 (50 ml), at a flow rate of 9 ml/h. Fractions (1.7 ml) were collected and their A_{280} values measured.

Dnp-albumins (1.5 ml, 25 mg/ml) were digested and chromatographed on DEAE-cellulose DE-52, under conditions identical with those given above. Before

chromatography the digest was dialysed against 0.02 M-sodium phosphate buffer, pH 6.0 (2 $\times$ 8 ml), and a measurement was made of the recovery of material absorbing at 280 and 360 nm. The A_{280} and A_{360} values of the column fractions were measured.

Anti-Dnp antibody fragments

We have described (Knight & Green, 1976) the isolation of the univalent fragments of high-affinity antibody to Dnp, and discussed their properties in fluorescence-quenching titrations with 6-*N*-Dnp-aminohexanoate. For difference-spectral titrations univalent fragments of antibody were isolated by the same method, omitting the final step of chromatography on Whatman CM-52 CM-cellulose.

Fluorescence-quenching titrations

Fluorescence-quenching titrations were carried out at 25°C in the apparatus described by Green (1967). The antibody concentration was 0.1–0.3 μM (with respect to binding sites) and the concentration of Dnp derivative was 20–50 μM in *N*-Dnp groups. Titrations were carried out in 0.05 M-sodium phosphate buffer, pH 8.0, or in 0.2 M-sodium acetate buffer, pH 3.0. The Dnp derivative was added in small portions until the antibody fluorescence was fully quenched, or until the corrections for internal quenching became too large. A correction curve for internal quenching by the Dnp group was made by adding portions of 6-*N*-Dnp-aminohexanoate (20 μM) to *N*-acetyltryptophan amide (1 μM) in 0.05 M-sodium phosphate buffer, pH 8.0. The quenching effect of increasing A_{280} values was determined by measuring the self-quenching of *N*-acetyltryptophan amide in the same buffer in the absorbance range up to $A_{280}^{1\text{cm}} = 0.1$. The quenching corrections were assumed to be identical at pH 3.0. Corrections were made for dilution during the titration.

Spectrophotometric titrations

The combination of the antibody-fragment preparations with 6-*N*-Dnp-aminohexanoate and with Dnp-bovine serum albumin was measured also by a modification of the spectrophotometric method of Little & Eisen (1967). The decrease in the value of the Dnp absorbance on adding the anti-Dnp antibody was measured at approx. 348 nm. Measurements were made in a Unicam SP. 700 double-beam recording spectrophotometer at 21°C in 0.05 M-sodium phosphate buffer, pH 8.0. Tandem cuvettes were inserted in each spectrophotometer compartment, one of each pair containing *N*-Dnp-albumin (0.35 ml, approx. 50 μM in Dnp groups) and the other buffer

(0.35 ml). The baseline (*N*-Dnp-albumin minus *N*-Dnp-albumin) was recorded between 300 and 625 nm. Portions (10 μ l) of antibody-fragment solution (0.48 mM in Dnp-binding sites) were added to the *N*-Dnp-albumin solution in the sample beam and to the buffer on the reference side. Buffer (10 μ l) was added to the *N*-Dnp-albumin solution in the reference beam. The contents of the cuvettes were mixed by gentle agitation. The observed absorption differences were corrected for dilution during the titration. When there was no further decrease in the A_{348} (after the addition of approx. 70 μ l of antibody solution) the difference spectrum was run over the original baseline. The spectra of antibody-bound and free *N*-Dnp-albumins were also recorded. Titrations with 6-*N*-Dnp-aminoheptanoate were carried out in the same way, but the absorbance change was followed at 356 nm. The results are expressed as $\Delta A/A_{363}$, where ΔA is the absorbance decrease at the minimum of the difference spectrum.

Results

Reaction of bovine serum albumin with 1-fluoro-2,4-dinitrobenzene

The reaction between bovine serum albumin (11.4 μ M) and 1-fluoro-2,4-dinitrobenzene (9.2 mM) was studied under pseudo-first-order conditions at pH 8.5 and 25°C. After 4 h 37 of the 60 potentially reactive amino groups had been modified and the reaction rate, measured by the increase in A_{400} , had become very slow. To convert data for A_{400} into molarities, or in this case into number of residues reacting, it was necessary to assume an average absorption coefficient for all the *N*-Dnp groups. The value 9000 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ is the average for 6-*N*-Dnp-lysine and 6-*N*-Dnp-lysine hydantoin. The initial reaction was analysed in terms of three sets of reactive amino groups. The pseudo-first-order

rate constants and numbers of amino groups in each set are shown in Table 1. The intrinsic pseudo-first-order rate constants for reaction with the unprotonated amino groups were calculated assuming that the average pK_a of the amino groups was 10.5 [the value determined by Freedman & Radda (1968) for α -*N*-acetyl-L-lysine amide].

If we include the residues in the slowest-reacting group there are at least four classes of reactive lysine residues in bovine serum albumin. Two residues react rapidly, as observed by Green (1963), but the majority react very slowly. It was difficult to measure the rate constant for the reaction of the least reactive lysine residues within the time scale of our experiments, but we estimate that these amino groups react approx. 30 times more slowly than the slowest kinetic set in Table 1. Thus the two lysine residues in the first kinetic set are approx. 1300 times more reactive.

Preparation and characterization of Dnp-albumins

Carboxymethylated bovine serum albumin reacted with 1 equivalent of 1-fluoro-2,4-dinitrobenzene to give a product whose absorption spectrum differed from that of 6-*N*-Dnp-lysine (Carsten & Eisen, 1953). The increased absorbance at 305 nm suggested the presence of *O*-Dnp-tyrosine, which has an absorption maximum at this wavelength (Sanger, 1949). Analysis of the spectrum showed approximately equal contents of 6-*N*-Dnp-lysine and *O*-Dnp-tyrosine. The reactive tyrosine residue was blocked by modification with di-isopropyl phosphorofluoridate (Shaw, 1962). Reaction of the carboxymethylated di-isopropyl-phosphoryl-protein with 1 equivalent of 1-fluoro-2,4-dinitrobenzene gave a product whose adsorption spectrum was very similar to that of 6-*N*-Dnp-lysine.

The Dnp-albumin preparations used in this work were all prepared from thiol-blocked di-isopropyl-phosphorylated albumin. Modification with di-isopropyl phosphorofluoridate protected the most reactive tyrosine from reaction with 1-fluoro-2,4-dinitrobenzene, but the spectra of Dnp-albumins with more than 1 mol of *N*-Dnp group/mol of albumin showed that the reaction was not confined to the lysine residues. The *O*-Dnp-tyrosine contents of a series of Dnp-albumins are shown in Table 2. Except at very low degrees of substitution approx. 30% of the protein-bound Dnp groups are present as *O*-Dnp-tyrosine. In the interpretation of our results we have assumed that the antibody binds only to *N*-Dnp groups. The antibody was raised against Dnp- γ -globulin containing exclusively *N*-Dnp groups (Eisen, 1964), and the dissociation constant for the interaction of *O*-Dnp-tyrosine with such antibody is approximately two orders of magnitude greater than that of 6-*N*-Dnp-lysine (Velick *et al.*, 1960; Eisen & Siskind, 1964).

Table 1. *Reactivities of amino groups in bovine serum albumin towards 1-fluoro-2,4-dinitrobenzene*

The rate constants were determined under pseudo-first-order conditions at pH 8.5 and 25°C as described in the Materials and Methods section. The intrinsic pseudo-first-order rate constants for reaction with the unprotonated amino group were calculated by assuming a value of 10.5 for the pK_a .

No. of groups in kinetic set	$10^4 \times$ Pseudo-first-order rate constants (s^{-1})	Intrinsic pseudo-first-order rate constants (s^{-1})
2	173	1.73
7	23	0.23
19	3.8	0.038

Table 2. *Spectral properties of Dnp-albumins and contents of 6-N-Dnp-lysine and O-Dnp-tyrosine*

The absorption spectra of the Dnp-albumins were recorded and the numbers of mol of 6-N-Dnp-lysine residues/mol of albumin calculated from A_{363} . The contribution of O-Dnp-tyrosine to the A_{305} was calculated as described in the Materials and Methods section. In the absence of O-Dnp-tyrosine the value of the ratio A_{305}/A_{363} would decrease from the values observed with the *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins to that observed with 6-N-Dnp-lysine hydantoin.

Compound	A_{305}/A_{363}	
6-N-Dnp-lysine hydantoin	0.12	
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)succinimidomercaptoethanol	0.10	
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)succinimidomercaptalbumin	0.26	
<i>N</i> -(<i>N</i> -Dnp-aminobutyl)succinimidomercaptalbumin	0.27	
<i>N</i> -(<i>N</i> -Dnp-aminoethyl)succinimidomercaptalbumin	0.28	

Residues of 6-N-Dnp-lysine/ molecule of albumin	A_{305}/A_{363}	Residues of O-Dnp-tyrosine/ molecule of albumin
0.95	0.34	0.07
1.85	0.35	0.36
3.2	0.39	1.2
4.4	0.41	1.6
5.4	0.41	2.2
7.8	0.42	3.3
9.0	0.45	4.8
15	0.41	6.6
24	0.49	14
31	0.44	13

Fig. 1. *Fluorescence of Dnp-albumins relative to N-acetyltryptophan amide at the same molar concentration*

Dnp-albumins were prepared as described in the Materials and Methods section. Fluorescence was measured in 0.05 M-sodium phosphate buffer, pH 8.0, at 25°C, relative to N-acetyltryptophan amide (AcTrpNH₂). The preparations of univalent fragments of antibody to Dnp used in this work had values of relative fluorescence in the range 4.5–5.5.

the corrected antibody fluorescence was quenched (Fig. 2). The initial slopes of the fluorescence-quenching titration curves obtained with *N*-Dnp-albumins had values much smaller than that observed with 6-N-Dnp-aminohexanoate under the same experimental conditions (Fig. 2).

Two series of experiments were carried out by using different preparations of univalent antibody fragments. The first series used antibody produced after only two doses of Dnp- γ -globulin. That used in the second series came from the same rabbits that had been given a third injection of antigen. The third injection resulted in greatly increased concentrations of antibody in the serum and an approx. 10-fold increase in affinity towards the hapten 6-N-Dnp-aminohexanoate.

As discussed previously (Knight & Green, 1976), the proportion of protein-bound Dnp groups binding to the antibody was determined by comparing the corrected initial slope of the titration curve with that of a titration with 6-N-Dnp-aminohexanoate at the same antibody concentration (Fig. 2). The fraction of available Dnp groups and the apparent dissociation constants, $K_{app.}$, of the Dnp-albumin-antibody complexes are given in Table 3.

In both series of experiments only a small proportion of the protein-bound *N*-Dnp groups were

Fluorescence of *N*-Dnp-albumins

The extent of quenching of the tryptophan fluorescence of bovine serum albumin at pH 8.0 by increasing numbers of mol of Dnp groups/mol of protein is shown in Fig. 1. The first Dnp group quenched approx. 70% of the protein fluorescence. In titrations of anti-Dnp antibody fragments with Dnp-albumins the residual albumin fluorescence led to an accompanying change in fluorescence opposite in direction to the quenching of the antibody fluorescence. Corrections were made as described previously (Knight & Green, 1976). At pH 3.0 the residual fluorescence of the Dnp-albumins was increased approx. 2-fold. When the albumin molecule bound more than 5 mol of Dnp groups/mol the tryptophan fluorescence was fully quenched at both pH values.

Titration of antibody with *N*-Dnp-albumins at pH 8.0

When the *N*-Dnp-albumins were added to univalent fragments of anti-Dnp antibody at pH 8.0

Fig. 2. Fluorescence quenching of univalent fragments of anti-Dnp antibody by 6-*N*-Dnp-aminohexanoate and by Dnp-albumins

Titration were made at 25°C in 0.05M-sodium phosphate buffer, pH 8.0. 6-*N*-Dnp-aminohexanoate (□) or Dnp-albumins (20 μM in *N*-Dnp) were added to univalent fragments of anti-Dnp antibody (2 ml, 0.2 μM in Dnp-binding sites). Corrections were made for the fluorescence of the Dnp-protein, for dilution and for internal quenching as described in the Materials and Methods section. The Dnp-albumin titrations were made with preparations containing the following numbers of mol of Dnp group/mol of albumin: ●, 1.0; ■, 2.0; ▲, 7.8; ○, 15.

available to antibody. The antibody used in the second experiment bound a higher proportion of the Dnp groups. As described previously (Knight & Green, 1976), most of the antibody in this preparation was of very high affinity. The fraction of groups available rose when the number of mol of Dnp/mol of albumin was increased, but did not exceed one-third. These results may be compared with the behaviour of the *N*-(*N*-Dnp-aminoalkyl)-succinimidomercaptalbumins (Knight & Green, 1976), where the Dnp groups were fully available to both antibody preparations even when the alkyl chain was short.

When there were less than approx. 8 mol of Dnp group/mol of albumin the apparent dissociation constants, $K_{app.}$, could not be determined. The method of calculation (Green & Work, 1953; Knight & Green, 1976) requires the value of $Q_{max.}$, the maximum observed degree of quenching of the antibody fluorescence in the presence of excess of antigen. At low degrees of substitution the values of $Q_{max.}$ could not be determined experimentally, as the

Table 3. Availability of Dnp groups of Dnp-albumins to univalent fragments of anti-Dnp antibody

Availability is defined as the ratio of the initial slope of a fluorescence-quenching titration with Dnp-protein to the initial slope of a titration with 6-*N*-Dnp-aminohexanoate at the same antibody concentration. Titrations were carried out at 25°C in 0.05M-sodium phosphate buffer, pH 8.0. Two independent experiments were made by using different preparations of univalent fragments of anti-Dnp antibody as described in the text. In fluorescence-quenching titrations with 6-*N*-Dnp-aminohexanoate the first antibody preparation had $K_{app.} = 15$ nM; the second preparation had $K_{app.} = 3.6$ nM. Some apparent dissociation constants, $K_{app.}$, are also given. Where no value of $K_{app.}$ is given the value of $Q_{max.}$ could not be determined experimentally.

Expt. 1

No. of mol of <i>N</i> -Dnp groups/mol of albumin	Availability	$10^8 \times K_{app.}$ (M)
1.4	0.02	—
2.0	0.02	—
3.7	0.07	—
5.0	0.09	—
6.5	0.13	—
9.3	0.14	2.7
15	0.14	3.8
19	0.14	3.8
24	0.17	2.7
28	0.19	1.9

Expt. 2

No. of mol of <i>N</i> -Dnp groups/mol of albumin	Availability	$10^8 \times K_{app.}$ (M)
1.0	0.07	—
2.0	0.08	—
4.4	0.09	—
7.8	0.20	—
9.3	0.25	2.3
15	0.25	1.8
24	0.26	1.9
31	0.32	1.8
36	0.32	2.0

corrections for the residual fluorescence of the Dnp-protein and for internal quenching increased with the volume of titrant. In our studies of the binding of *N*-(*N*-Dnp-aminoalkyl)succinimidomercaptalbumins to anti-Dnp antibody (Knight & Green, 1976) we used the value of $Q_{max.}$ observed in titrations with 6-*N*-Dnp-aminohexanoate to calculate the values of $K_{app.}$. In the titrations with *N*-Dnp-albumins this method was inappropriate, because the protein-bound Dnp groups did not behave like the free hapten with respect to quenching efficiency. The values of $Q_{max.}$ observed in titrations with *N*-Dnp-albumins were approx. 0.55 in the range 9–19 mol of Dnp group/mol of protein, increasing to approx.

0.78 at the highest degrees of substitution. In titrations with 6-(*N*-Dnp)amino hexanoate the value of $Q_{\max}$ was 0.85 (Knight & Green, 1976).

Titration of antibody with N-Dnp-albumins at pH 3.0

The majority of *N*-Dnp groups on Dnp-albumin were unavailable to antibody fragments at pH 8.0. The failure to bind antibody could be due to grouping of the Dnp groups in clusters such that an antibody fragment bound to one Dnp group masked the neighbouring groups. This argument cannot explain the inaccessibility of the Dnp group on mono-Dnp-albumin. An alternative explanation is that antibody binding was restricted by location of the Dnp group at the van der Waals' surface of the protein. In this case efficient binding would be prevented by steric hindrance (Green, 1969).

If the initial reaction of 1-fluoro-2,4-dinitrobenzene occurs at lysine residues located in the non-polar anion-binding sites, as suggested by Green (1963), the accessibility of hindered Dnp groups to antibody may be increased at acid pH values. Foster (1960) has shown that in the acid-induced unfolding of serum albumin the strong binding sites for non-polar anions are lost.

The results of fluorescence-quenching titrations comparing the quenching efficiency of bovine serum albumin-bound Dnp groups at pH 8.0 and pH 3.0 are given in Table 4. Titrations at the two pH values were carried out consecutively. The experiments shown in Table 4 used the same high-affinity antibody as the second series in Table 3. The antibody was titrated with 6-*N*-Dnp-amino hexanoate at each pH value and showed no decrease in affinity for the hapten on lowering the pH (Velick *et al.*, 1960). The fraction of available Dnp groups was calculated from the initial slopes of the titration curves as described above. The initial portion of the corrected titration curves obtained with approx. 1 Dnp group/mol of albumin showed considerable curvature at pH 3.0,

but was linear at pH 8.0. The reason for this behaviour is unknown and the initial portions of the titration curves were linear at both pH values at higher degrees of substitution.

The influence of pH on the accessibility of the protein-bound Dnp groups to antibody depended on the number of mol of Dnp group/mol of albumin. It was observed that at low degrees of substitution the fraction of available Dnp groups increased approx. 3-fold when the pH value was lowered from pH 8.0 to 3.0. As the number of protein-bound Dnp groups/mol increased, the effect of acid pH values on the availability of the protein-bound Dnp groups decreased. The apparent dissociation constants could not be measured at either pH value.

The effect of pH on the accessibility of Dnp groups in Dnp-albumins with a small number of mol of Dnp group/mol is consistent with the model that places the Dnp groups on lysine residues in clefts between globular sub-domains of the single polypeptide chain. In the structural transition at acid pH values these sub-domains separate (Harrington *et al.*, 1956) and the Dnp groups become more accessible to the univalent fragments of anti-Dnp antibody.

Fragmentation of Dnp-albumins by pepsin

The results obtained in fluorescence-quenching titrations at pH 3.0 suggested that steric hindrance was the major factor preventing the combination of the antibody and albumin-bound Dnp groups. The influence of protein-protein interaction cannot, however, be overcome even in the more flexible pH 3.0 form of albumin. Bovine serum albumin can be cleaved into globular fragments by pepsin digestion (Weber & Young, 1964). We isolated Dnp-containing fragments of Dnp-albumin after pepsin digestion at pH 3.0 and examined the ability of the fragments to quench the fluorescence of anti-Dnp antibody.

A pepsin digest of bovine serum albumin, made as described by Weber & Young (1964), was applied to a column of Whatman DE-52 DEAE-cellulose. The chromatographic pattern showed three peaks of u.v.-absorbing material. The recovery of A_{280} units was approx. 70%. Weber & Young (1964) observed a similar elution pattern and showed that the first peak contained a fragment of mol.wt. 12000, whereas the fragments in the second and third peaks both had mol.wt. values of approx. 30000 and had similar amino acid compositions. A pepsin digest of Dnp-albumin with 1.0 mol of Dnp group/mol of albumin also gave three peaks of material absorbing at 280 nm (Fig. 3a). Some material absorbing at 360 nm was found in the first peak, but most of the Dnp groups were eluted in the second peak (Fig. 3a). The recovery of the original absorbance units from the DE-52

Table 4. *Availability of bovine serum albumin-bound Dnp groups to anti-Dnp antibody at pH 8.0 and 3.0*

Availability is defined as described in Table 3. Fluorescence-quenching titrations were carried out at 25°C in 0.05 M-sodium phosphate buffer, pH 8.0, and also in 0.2 M-sodium acetate buffer, pH 3.0. In titrations with 6-*N*-Dnp-amino hexanoate the antibody preparation had $K_{app} = 2.4$ nM at pH 8.0 and $K_{app} = 1.8$ nM at pH 3.0.

No. of mol of <i>N</i> -Dnp group/mol of albumin	Availability at pH 8.0	Availability at pH 3.0
2.0	0.12	0.43
4.4	0.14	0.46
7.5	0.20	0.46
9.3	0.27	0.48

Fig. 3. Chromatography on DE-52 DEAE-cellulose of pepsin digests of Dnp-albumins containing (a) 1.0 mol of Dnp group/mol of albumin and (b) 4.9 mol of Dnp group/mol of albumin

Dnp-albumins (1.5 ml, 25 mg/ml) were digested with pepsin (0.1%, w/w) at pH 3.0 for 70 min at 25°C. After dialysis against 0.02 M-sodium phosphate buffer, pH 6.0 (2 × 8 ml), the digest was applied to a column (0.8 cm × 11 cm) of Whatman DE-52 DEAE-cellulose. The column was eluted at 4°C with a linear gradient of sodium phosphate buffer, pH 6.0, from 0.02 M (50 ml) to 0.33 M (50 ml). The flow rate was 9 ml/h. Fractions (1.7 ml) were collected and their A_{280} (—) and A_{360} (---) values measured.

DEAE-cellulose column was 66% at 280 nm and 69% at 360 nm. The digest has been dialysed before application to the column and the diffusate contained 12% of the original A_{280} units, but less than 3% of the A_{360} units. The absorbance units not recovered may have been associated with a strongly

adsorbed band of Dnp-protein, which remained at the top of the column.

Pepsin digests were made of Dnp-albumins with up to 5 mol of Dnp group/mol of albumin. At the highest degree of substitution the elution pattern from the DE-52 DEAE-cellulose column showed a broad second peak of material absorbing at 280 nm (Fig. 3b). The leading edge of this peak contained those fractions having the maximum absorbance at 360 nm (Fig. 3b).

Titration of antibody with fragments of Dnp-albumins

When Dnp-albumin carrying 1.0 mol of Dnp group/mol of albumin was added to antibody the initial fluorescence decrease was much less than that observed in a titration with 6-*N*-Dnp-amino-hexanoate at the same Dnp concentration (Fig. 4). Chromatography of a pepsin digest of this Dnp-albumin preparation gave the elution profile shown in Fig. 3(a). A fluorescence-quenching titration was made with the Dnp-labelled fragment from the fraction showing the maximum A_{360}/A_{280} ratio. The Dnp-fragment quenched the antibody fluorescence with an efficiency only slightly less than that of the free hapten (Fig. 4).

Fluorescence-quenching titrations were made with three preparations of Dnp-albumin and their Dnp-fragments separated on DE-52 DEAE-cellulose. In each case the proportion of protein-bound Dnp groups binding to the antibody was determined by comparing the corrected initial slope of the titration with 6-*N*-Dnp-amino-hexanoate at the same antibody concentration. The fraction of available Dnp groups and the apparent dissociation constants are shown in Table 5. The results show that Dnp groups bound to native albumin have a low quenching efficiency. On pepsin digestion the quenching efficiency of the protein-bound Dnp groups increases to that of the free hapten and so the Dnp groups must be in exposed positions, allowing free access of the univalent fragments of anti-Dnp antibody. The apparent dissociation constants are significantly greater than that of the hapten 6-*N*-Dnp-amino-hexanoate.

Difference-spectral titrations at pH 8.0

The results of the fluorescence-quenching titrations at pH 8.0 have been interpreted in terms of two classes of Dnp group that may be called 'buried' and 'exposed' on the basis of their accessibility to antibody (Knight & Green, 1976). However, it is not possible to distinguish from these experiments between this interpretation and an alternative model with all the Dnp groups equally accessible to antibody, but with an increased dissociation constant. If this were the case the availability would be merely the proportion of Dnp groups bound under the

Fig. 4. Fluorescence-quenching of univalent fragments of anti-Dnp antibody by the Dnp-containing fragment isolated from mono-Dnp-albumin after pepsin digestion

Dnp-albumin containing 1 mol of Dnp group/mol was digested with pepsin, and the Dnp-containing fragment isolated as described in the legend to Fig. 3. A fluorescence-quenching titration was carried out with the Dnp-containing fragment (■) as described in the legend to Fig. 2. Titrations of the antibody with 6-*N*-Dnp-aminohexanoate (▲) and the undigested mono-Dnp-albumin (●) are also shown.

Table 5. Availability to antibody of the Dnp groups of native Dnp-albumin and of the Dnp-containing fragments isolated after pepsin digestion

Dnp-albumins were digested with pepsin, and the Dnp-containing fragments isolated by chromatography on DE-52 DEAE-cellulose as described in the Materials and Methods section. The availability of the protein-bound Dnp groups to antibody was measured by fluorescence-quenching titrations at 25°C in 0.05M-sodium phosphate buffer, pH 8.0. In titrations with 6-*N*-Dnp-aminohexanoate the antibody preparation had $K_{app.} = 1.7$ nm.

Dnp-albumin		Dnp-fragment	
No. of mol of Dnp group/mol of albumin	Availability	Availability	$10^8 \times K_{app.}$ (M)
1.0	0.07	0.92	1.2
3.9	0.11	1.01	1.7
4.9	0.17	1.02	1.3

initial conditions of antibody excess in the fluorescence-quenching titrations. We have calculated the proportion of Dnp bound by a 10-fold molar excess of antibody assuming different values of the apparent dissociation constant. In a fluorimetric titration where the antibody concentration was

0.2 μM only 10% of the Dnp groups would appear exposed if the system could be described by a single dissociation constant having the value 2.5 μM. If the concentration of both antibody and Dnp were about 80 μM, over 90% of the Dnp groups would become antibody-bound. We have studied therefore the proportion of Dnp bound at high antibody concentrations (80 μM) by a modification of the spectral-shift method of Little & Eisen (1967).

In the fluorescence-quenching titrations the Dnp compound was added to an excess of univalent fragments of antibody to Dnp and the initial slope measured the proportion of Dnp groups binding. By contrast, in the spectrophotometric titrations the Dnp compound was initially in excess. The initial slope of the plot of $\Delta A_{348}/A_{363}$ against antibody concentration measured the proportion of specific antibody in the antibody preparation that combined with albumin-bound Dnp groups. The proportion of Dnp groups available to the antibody was determined from the maximum decrease in A_{348} relative to that given by the hapten. We observed that titrations with 6-*N*-Dnp-aminohexanoate always gave higher values of the initial slope compared with comparable titrations with Dnp-albumins, although within a series of Dnp-albumin titrations the initial slopes were very similar in value. These differences in slope probably reflect the presence of antibodies with low affinity for albumin-bound Dnp groups, which nevertheless bind to the free hapten. The end points of the titrations were not affected, since they correspond to conditions of antibody excess.

Dnp difference spectra were recorded at the end points of titrations of anti-Dnp antibody with 6-*N*-Dnp-aminohexanoate and Dnp-albumins. All the difference spectra recorded for 6-*N*-Dnp-aminohexanoate had minima at 356 nm; the Dnp-albumin difference-spectral minima were in the region 348 ± 2 nm. Little & Eisen (1967) reported that the difference spectrum of the antibody-bound Dnp group had maxima at 390 ± 2 nm and 470 ± 3 nm. We observed maxima in the difference spectra at approx. 393 nm and 475 nm, in good agreement with the values found by Little & Eisen (1967). We have assumed that if the protein-bound Dnp groups were fully available to antibody the value of $\Delta A/A_{363}$ in the region of 348 nm would have the same value as that observed at 356 nm in a titration with 6-*N*-Dnp-aminohexanoate. Thus the availability of the Dnp groups is defined as the ratio of the values of $\Delta A/A_{363}$ observed in titrations with Dnp-albumins and with 6-*N*-Dnp-aminohexanoate. Difference-spectral titrations were made with a series of Dnp-albumins, and the fraction of available Dnp groups is shown in Table 6. Fluorescence-quenching titrations were made with the same antibody and Dnp-albumins. The availabilities of the Dnp groups determined by this method are also shown in Table 6.

Table 6. *Comparison of the availability to antibody of albumin-bound Dnp groups determined by difference-spectral and fluorescence-quenching titrations*

Difference-spectral titrations were made at 21°C in 0.05M-sodium phosphate buffer, pH 8.0, as described in the Materials and Methods section. The binding of antibody to Dnp-albumin was followed by the absorbance decrease at approx. 348nm. The maximum absorbance difference at antibody excess was normalized by dividing by the initial Dnp absorbance at 363nm. Titrations were also made with 6-*N*-Dnp-aminohexanoate, by following the absorbance decrease at 356nm, and normalizing the maximum absorbance difference. The availability of the protein-bound Dnp groups is defined as the ratio of the normalized absorbance differences observed for the Dnp-protein and for 6-*N*-Dnp-aminohexanoate, as described in the text. The availability of the protein-bound Dnp groups was also determined by fluorescence-quenching titrations as described in Table 3.

No. of mol of Dnp group/mol of albumin	Availability	
	By difference- spectral titration	By fluorescence- quenching titration
1.0	0.32	0.12
1.9	0.56	0.11
3.2	0.61	0.12
5.3	0.44	0.19
6.9	0.37	0.19
8.3	0.37	0.20
9.3	0.51	0.26

These results show that when bovine serum albumin carried an average of 1 mol of Dnp group/mol the major fraction of the Dnp groups is not freely accessible to antibody, even at antibody concentrations of 80 μ M. The titrations with Dnp-albumins carrying 1.9 and 3.2 mol of Dnp group/mol showed that increasing the concentration of antibody led to an increase in the availability of the Dnp groups, but that one Dnp group remained inaccessible to antibody. For albumin molecules carrying more than 5 mol of Dnp/mol, fluorescence-quenching titrations gave values for the number of available groups that were about half of those determined by the difference-spectral titrations. It appears, therefore, as might have been expected, that although some Dnp groups are almost totally inaccessible to antibody others merely have their dissociation constant increased by 2-4 orders of magnitude.

Discussion

The kinetic study of the reaction of 1-fluoro-2,4-dinitrobenzene with lysine residues of bovine serum albumin confirms the observation by Green (1963) that albumin contains two residues that react

rapidly. Human serum albumin also contains two lysine residues highly reactive towards arenesulphonic acids and aryl halides (Goldfarb, 1966; Gerig & Reinheimer, 1975). The rate of reaction observed for the most reactive lysine residue is approx. 500 times faster than the rates of reaction of low-molecular-weight lysine derivatives with 1-fluoro-2,4-dinitrobenzene (Brouwer *et al.*, 1958; Shivaram & Wallenfels, 1969; Ghazarian, 1972). The high reactivity could be a consequence of the local binding of the reagent (Green, 1963; Gerig & Reinheimer, 1975), but there is also evidence that the most reactive lysine residues in human serum albumin have an unusually low value of pK_a (7.9; Gerig & Reinheimer, 1975). Although such a decrease in pK_a could be the consequence of a hydrophobic environment (Schmidt & Westheimer, 1971), we cannot rule out other factors such as the influence of local positive charge, especially in view of the clustering of basic residues in the sequence (Brown, 1977).

The spectroscopic properties of albumin-bound Dnp groups are independent of the number of Dnp groups/molecule of protein, although the absorption spectrum of the Dnp group is sensitive to changes in the polarity of its environment (Eisen & Siskind, 1964; Ettinger & Hirs, 1968). Moreover, in solvent perturbation studies with methanol (C. G. Knight & N. M. Green, unpublished work) the albumin-bound Dnp groups behaved similarly to the free hapten. The protein-bound Dnp groups appear therefore to be fully exposed to the solvent.

However, fluorescence-quenching titrations showed that Dnp groups attached via lysine residues to native serum albumin were largely unavailable to univalent fragments of high-affinity antibody to Dnp (Table 3). The apparent dissociation constants for the protein-bound Dnp groups could not be measured at low degrees of substitution. These results suggested that the hindrance to the binding of antibody by Dnp-albumin arose from protein-protein interactions.

The unfolding of the albumin molecule at acid pH values (Foster, 1960) results in a marked increase in the ability of the Dnp-protein to bind antibody (Table 4), but the apparent dissociation constants remained too high to be measured under the conditions of the fluorescence-quenching titrations. The globular sub-domains of serum albumin can be separated by digestion with pepsin (Weber & Young, 1964; King, 1973; Braam *et al.*, 1974; Feldhoff & Peters, 1975; Geisow & Beaven, 1977). The major Dnp-containing fragments isolated by chromatography resembled the free hapten, 6-*N*-Dnp-amino-hexanoate, in their ability to bind antibody (Fig. 4 and Table 5). Hence proteolytic cleavage produced protein-bound Dnp groups whose antibody-binding properties resembled those of *N*-(*N*-Dnp-amino-

alkyl)succinimidomercapalbumins (Knight & Green, 1976), and so we conclude that the Dnp groups on the fragments are free to move outside the van der Waals' surface of the protein. The progressive increase in the availability of the albumin-bound Dnp groups to antibody as the pH value was lowered and the unfolded form of the protein digested supports the model that locates the reactive lysine residues in clefts between the globular sub-domains of the albumin molecule (Green, 1963).

Unfortunately, there is not enough information about the fragments obtained by Weber & Young (1964) to identify their location in the albumin molecule. However, kinetic studies by Taylor *et al.* (1975) have shown that one of the lysine residues of bovine serum albumin most reactive towards 1-fluoro-2,4-dinitrobenzene is blocked by pyridoxal phosphate and that this is lysine-220 (Taylor, 1976), near the beginning of the second of the three globular domains (Brown, 1977). The other reactive lysine residues are probably in other domains; for example, Brown (1977) has shown that lysine-410 is preferentially labelled by trinitrobenzenesulphonic acid. Current models of the structure of albumin (Brown, 1977) place many of the lysine residues in groups in the bends joining the ends of helical rods. This clustering may be responsible in part for the apparent low availability of the Dnp groups in the more highly substituted derivatives, but it cannot account for the results obtained with derivatives containing less than 5 Dnp groups/molecule. The restricted access of antibody to the Dnp residues in these derivatives places limitations on acceptable models for the albumin structure. It also shows that the most reactive lysine residues are not those that are most exposed to the solvent (cf. Goldfarb, 1966).

The difference-spectral titrations are in agreement with the conclusion that some proportion of the protein-bound Dnp groups cannot bind antibody even at high concentrations of both reactants. The limited degree of binding observed at 1 mol of Dnp group/mol of albumin probably arose from the unavoidable heterogeneity in both extent and site of reaction. When each mol of albumin contained approx. 2 and 3 mol of Dnp group (Table 6) one group remained inaccessible to antibody. These results suggested that the second and third Dnp groups were only partially buried and that some of the effects of steric hindrance could be overcome at the relatively high protein concentrations employed in the difference-spectral titrations. The Dnp groups became fully available to antibody after proteolytic cleavage of the Dnp-albumin (Table 5), implying that binding to the native Dnp-protein was hindered by the adjacent surface of the albumin molecule.

The precise measurement of the accessibility of the amino acid residues contributing to the van der Waals'

surface of a protein has been reviewed by Richards (1977). These studies required the detailed three-dimensional structure of the protein, derived from X-ray crystallography, to be known. A less-demanding method of measuring exposure, applicable to aromatic chromophores, is that of solvent perturbation spectroscopy (Herskovits & Laskowski, 1962). These theoretical and practical studies have demonstrated the same conclusion, namely that the definition of a residue as buried or exposed depends on the dimensions of the molecule used to probe the surface. By spectral criteria the Dnp groups attached to the most reactive lysine residues of bovine serum albumin are fully exposed, but they are inaccessible to univalent fragments of high-affinity antibody to Dnp, a macromolecular probe. By contrast, the Dnp group of [Dnp-Lys⁴¹]ribonuclease A, which can be classified as partially buried by both crystallographic (Allewell *et al.*, 1973) and spectral criteria (Ettinger & Hirs, 1968), bound anti-Dnp antibody (Knight & Green, 1976). These studies indicate that when proteins can be specifically labelled with *N*-Dnp groups the binding of univalent fragments of antibody to Dnp provides a useful additional indicator of the local environment of the Dnp group and its conformational flexibility.

References

- Allewell, N. M., Mitsui, Y. & Wyckoff, H. M. (1973) *J. Biol. Chem.* **248**, 5291–5298
- Baldwin, R. L. (1957) *Biochem. J.* **65**, 503–512
- Braam, W. G. M., Hilak, M. C., Harmsen, B. J. M. & Van Os, G. A. J. (1974) *Int. J. Peptide Protein Res.* **6**, 21–29
- Brouwer, D. M., Van der Vlugt, M. J. & Havinga, E. (1958) *Proc. Ned. Akad. Wet. Ser. B* **61**, 141–149
- Brown, J. R. (1977) *Proc. FEBS Meet. 11th, Copenhagen* (Peters, T., Jr., ed.), vol. 50, pp. 1–10, Pergamon, Oxford
- Carsten, M. E. & Eisen, H. N. (1953) *J. Am. Chem. Soc.* **75**, 4451–4456
- Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181
- Eisen, H. N. (1964) *Methods Med. Res.* **10**, 94–102
- Eisen, H. N. & Siskind, G. W. (1964) *Biochemistry* **3**, 996–1008
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Ettinger, M. J. & Hirs, C. H. W. (1968) *Biochemistry* **7**, 3374–3380
- Feldhoff, R. C. & Peters, T., Jr. (1975) *Biochemistry* **14**, 4508–4514
- Fletcher, J. E., Spector, A. A. & Ashbrook, J. D. (1970) *Biochemistry* **9**, 4580–4587
- Foster, J. F. (1960) in *The Plasma Proteins* (Putnam, F. W., ed.), vol. 1, pp. 179–239, Academic Press, New York and London
- Freedman, R. B. & Radda, G. K. (1968) *Biochem. J.* **108**, 383–391
- Geisow, M. J. & Beaven, G. H. (1977) *Biochem. J.* **161**, 619–625

- Gerig, J. T. & Reinheimer, J. D. (1975) *J. Am. Chem. Soc.* **97**, 168–173
- Ghazarian, J. G. (1972) *Arch. Biochem. Biophys.* **150**, 72–76
- Goldfarb, A. R. (1966) *Biochemistry* **5**, 2574–2578
- Goodman, D. S. (1958) *J. Am. Chem. Soc.* **80**, 3892–3898
- Green, N. M. (1963) *Biochim. Biophys. Acta* **74**, 542–543
- Green, N. M. (1967) *Biochem. J.* **105**, 54p
- Green, N. M. (1969) *Adv. Immunol.* **11**, 1–30
- Green, N. M. & Work, E. (1953) *Biochem. J.* **54**, 347–352
- Harrington, W. F., Johnson, P. & Ottewill, R. H. (1956) *Biochem. J.* **62**, 569–582
- Herskovits, T. T. & Laskowski, M., Jr. (1962) *J. Biol. Chem.* **237**, 2481–2492
- Hughes, W. L., Jr. (1947) *J. Am. Chem. Soc.* **69**, 1836–1837
- King, T. P. (1973) *Arch. Biochem. Biophys.* **156**, 509–520
- Knight, C. G. & Green, N. M. (1976) *Biochem. J.* **159**, 323–333
- Little, J. R. & Eisen, H. N. (1967) *Biochemistry* **6**, 3119–3125
- Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng.* **6**, 151–176
- Sanger, F. (1949) *Biochem. J.* **45**, 563–574
- Schmidt, D. E., Jr. & Westheimer, F. H. (1971) *Biochemistry* **10**, 1249–1253
- Shaw, D. C. (1962) Ph.D. Thesis, University of Cambridge
- Shivaram, K. N. & Wallenfels, K. (1969) *Indian J. Biochem.* **6**, 77–81
- Stark, G. R. & Smyth, D. G. (1963) *J. Biol. Chem.* **238**, 214–226
- Taylor, R. P. (1976) *J. Am. Chem. Soc.* **98**, 2684–2686
- Taylor, R. P., Chau, V., Bryner, C. & Berga, S. (1975) *J. Am. Chem. Soc.* **97**, 1934–1943
- Tombs, M. P., Souter, F. & MacLagan, N. F. (1959) *Biochem. J.* **73**, 167–171
- Velick, S. F., Parker, C. W. & Eisen, H. N. (1960) *Proc. Natl. Acad. Sci. U.S.A.* **46**, 1470–1482
- Weber, G. & Young, L. B. (1964) *J. Biol. Chem.* **239**, 1424–1431