The Problem of Hapten Persistently Bound to Antibody

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A common schedule for purifying antibodies to haptens entails dissolving a specific precipitate with concentrated hapten, removing the freed macromolecular antigen and finally removing the hapten. In the last step it is often extremely difficult to remove all the bound hapten (Eisen, 1964; Stevenson & Stevenson, 1967). Ideally, a chromatographic procedure such as passage through dextran gel (Sephadex) should continually remove free hapten from the environment of antibody molecules, with a progressive decrease in the occupancy of antibody sites as the equilibrium:

is displaced to the left. Ultimately the antibody should be essentially hapten-free. Two mechanisms could contribute to a failure to achieve this. (a) The rate constant k_{-1} might be so small that many complexes never dissociate during the chromatographic procedure; the survival of such complexes would be independent of the value to which the concentration of free hapten is lowered. (b) The chromatographic procedure might be incapable of depleting the antibody of vicinal free hapten to an extent at which antibodies of high affinity, at equilibrium, have their sites largely unoccupied.

Knowledge of the relative contributions of these mechanisms should help the planning of purification procedures. Here we investigate their roles in the cases of anti-DNP† and anti-ABS. The respective haptens used were ϵ -DNP-lysine and 4'-dimethylaminoazobenzene-4-sulphonate (Methyl Orange). Attempts were made to strip these haptens from their antibodies by passage through columns of Sephadex. By estimating at the same time the rate of exchange of radioactive hapten between free and bound forms we have been able to evaluate the contribution of mechanism (a) to the inefficiency of the Sephadex.

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† Abbreviations: anti-DNP, antibody to the 2,4-dinitrophenyl determinant; anti-ABS, antibody to the azobenzene-4-sulphonate determinant.

All experiments were at room temperature. The buffer throughout was 0.2M-tris-HCl, pH 8.0. Column effluents were monitored with an LKB Uvicord spectrophotometer. β -emission was measured in a Unilux II counter (Nuclear-Chicago). The scintillant was toluene-Triton X-100 adapted for counting in homogeneous solution (Turner, 1967), with the aqueous content maintained strictly at 10%. Counting efficiencies were estimated from standards containing a range of concentrations of the corresponding hapten. Control experiments with rabbit non-specific immunoglobulin G always revealed less than 5% of the binding shown by antibody.

Anti-DNP. Antibodies of high affinity were raised in rabbits and purified from pooled serum by one of the methods described by Eisen, Gray, Little & Simms (1967): precipitation with trinitrophenylated human serum albumin, dissolution with 2,4-dinitrophenol and passage through a layered column of DEAE-cellulose on top of Dowex 1. By avoiding exposure to a strongly bound hapten this schedule yields antibody that is essentially hapten-free.

Antibody with persistently bound labelled hapten was now prepared. ϵ -[^3H]DNP-lysine, prepared as described by Eisen, Simms & Potter (1968), was mixed with carrier to give a final solution 0.15mM, 0.37 Ci/mol. Then 1 ml of this solution was added to 10 mg of anti-DNP in 1 ml of buffer. The solution was applied to a column (1.6 cm $\times$ 10 cm) of Sephadex G-25 (fine grade) equilibrated with the tris-HCl buffer. Even on such a small column antibody and free hapten migrated as apparently well-separated zones, so that hapten emerging with the antibody was regarded as 'persistently bound'. The middle 4 ml of the protein zone, which was delivered within 4 min of application to the column, was harvested. This gave a recovery of 74% based on E_{280} with an allowance for E_{280} of the bound hapten, and had a ^3H content of 8.46 nCi/ml. These results indicate a protein concentration of 1.85 mg/ml, with bound ϵ -DNP-lysine 1.86 mol/150 000 g of protein. The antibody-hapten solution was now treated in two parts.

(1) A 1 ml portion, containing 8.46 nCi of ^3H , was

Table 1. *Displacement of persistently bound hapten after various times of incubation with free hapten*

Anti-DNP with persistently bound ϵ -[^3H]DNP-lysine was incubated with unlabelled hapten at a concentration 87 times that of the bound hapten. After the times in column 1, 1 ml lots of the solution, containing 4.23 nCi of ^3H , were applied to a column (1.6 cm $\times$ 10 cm) of Sephadex G-25 (fine grade) and the antibody-hapten complexes delivered had their ^3H content determined (column 2). The proportion of the original persistently bound hapten that had exchanged with free hapten was given by $(4.23 - 100y)/(4.18 + 0.015y)\%$, where y is the ^3H content. It is assumed that no exchange occurred after application to the Sephadex, and that the persistently bound hapten was 1.86 mol/150 000 g of protein for all runs through the column. Anti-ABS with persistently bound unlabelled Methyl Orange was incubated with [^{35}S]Methyl Orange at a concentration 17 times that of the bound hapten. After the times indicated in column 1, 2 ml lots of the solution were applied to a column (1.6 cm $\times$ 10 cm) of Sephadex G-25 (fine grade) and the antibody-hapten complexes delivered had their ^{35}S content determined (column 2). The proportion of the original persistently bound hapten that had exchanged with free hapten was given by $100y/(2.75 - 0.058y)\%$, where y is the ^{35}S content. It is assumed that no exchange occurred after application to the Sephadex, and that the persistently bound hapten was 0.73 mol/150 000 g of protein for both runs through the column.

	Time of application to column	Radioactivity delivered with antibody by column (nCi)	Bound hapten exchanged by time of application to column (%)
Anti-DNP	5 min	1.30	70
	8 h	0.73	83
	24 h	0.52	88
Anti-ABS	5 min	2.35	90
	24 h	2.63	101

diluted with 1 ml of buffer and applied to a column (1.5 cm $\times$ 40 cm) of Sephadex G-25 (fine grade) equilibrated with the buffer. The flow was controlled by a peristaltic pump so that delivery of the protein zone began 25 h after application. The entire protein zone was collected and found to contain 8.21 nCi of ^3H . Thus during this second, prolonged, passage through Sephadex the antibody lost less than a further 5% of its persistently bound hapten.

(2) To 2 ml was added 2 ml of 2 mM unlabelled ϵ -DNP-lysine, decreasing the overall specific radioactivity of the hapten 88-fold. After standing for 5 min, 8 h and 24 h, 1 ml lots of this solution were put through the small column (1.6 cm $\times$ 10 cm) of Sephadex G-25 (fine grade). In each case the entire protein zone was harvested and its ^3H content estimated. One obtains thereby a measure of the rate at which bound and free hapten had exchanged;

any exchange occurring after application to the Sephadex was neglected, and it was assumed that the persistently bound hapten was 1.86 mol/150 000 g of protein for all runs through this column. The results (Table 1) show that the bound hapten had not exchanged completely by 5 min, and possibly not even by 24 h. Accordingly, during a passage through Sephadex lasting less than 24 h some complexes with hapten formed by this antibody do not dissociate, so that mechanism (a) contributes to the inefficiency of the Sephadex. But this contribution is clearly a minor one; approx. 90% of the complexes must have dissociated and exchanged their hapten within 24 h, whereas, from the previous experiment, continuous passage through Sephadex for 25 h decreased the extent of binding by less than 5%.

Anti-ABS. The antibody was evoked in rabbits and purified from pooled serum as described by Stevenson & Stevenson (1967). [^{35}S]Methyl Orange was prepared by coupling diazotized [^{35}S]sulphanilic acid (The Radiochemical Centre, Amersham, Bucks., U.K.) with *N,N*-dimethylaniline, and was recrystallized from hot water in the presence of carrier (final specific radioactivity 0.102 Ci/mol). The persistence of bound hapten was investigated in the same way as with anti-DNP, except that unlabelled hapten was displaced by labelled hapten because the preparative method had left an appreciable amount of the former on the antibody.

A standard amount of persistently bound hapten was established as before. A 2 ml portion of solution containing 18 mg of anti-ABS, made 0.25 mM with respect to Methyl Orange, was applied to a column (1.6 cm $\times$ 10 cm) of Sephadex G-25 (fine grade) equilibrated with the tris-HCl buffer. The middle 4.5 ml of the protein solution was harvested. This contained antibody at 3.6 mg/ml, with 0.73 mol of bound Methyl Orange/150 000 g. These values were calculated from $E_{280}^{1\text{cm}}$ 4.96 and $E_{465}^{1\text{cm}}$ 0.411, by using the following values for ϵ : protein, 200 000 at 280 nm, 0 at 465 nm; bound Methyl Orange, 9500 at 280 nm, 23 400 at 465 nm. Once again the solution was treated in two parts.

(1) A 1 ml portion was diluted with 1 ml of buffer and applied to the long column (1.5 cm $\times$ 140 cm) of Sephadex G-25 (fine grade). The antibody was delivered after 25 h and revealed $E_{280}^{1\text{cm}}$ 0.821 and $E_{465}^{1\text{cm}}$ 0.063, from which the bound Methyl Orange was 0.68 mol/150 000 g. Thus a further 7% of the persistently bound hapten had been removed.

(2) To 3.3 ml was added 1 ml of 1 mM- ^{35}S]Methyl Orange. Then 2 ml portions were put through the small column of Sephadex at 5 min and 24 h. In each run the antibody was delivered within 4 min, was harvested in its entirety and had its content of ^3H estimated. Again the results (Table 1) show that the failure of the long run through Sephadex to

remove persistently bound hapten can be attributed only to a very minor extent to the failure of antibody-hapten complexes to dissociate.

We have from time to time tried other chromatographic methods for removing hapten from anti-DNP and anti-ABS, for example passage through Dowex 1 or DEAE-cellulose. None has been obviously superior to Sephadex.

In summary, in two antibody-hapten systems the principal reason for the failure of chromatography on Sephadex to remove a considerable proportion of bound hapten appears to be a failure to decrease sufficiently the concentration of free hapten. A minor contribution arises from the failure of some antibody-hapten complexes to dissociate during the period of chromatography.

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