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Comparison of Immunoadsorbents Prepared by Coupling Sperm-Whale Myoglobin to a Variety of Insoluble Polymers

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Immunoadsorbents that were potentially suitable for the fractionation of antibodies to myoglobin were prepared by coupling myoglobin to aminoethylcellulose, bromoacetylcellulose, CM-cellulose, an ethylene–maleic anhydride co-polymer and Sepharose 4B. A comparison of the properties of the preparations indicated that Sepharose–myoglobin was superior, as an immunoadsorbent, to the other conjugates. Elution of adsorbed antibodies with M-propionic acid, pH 2.5, gave yields of precipitable antibody higher than those achieved with other dissociating solvents. Propionic acid eluted 60–100% of the antibody adsorbed by Sepharose–myoglobin, and 60–90% of the eluted protein was precipitable antibody.

Water-insoluble derivatives of protein antigens have been extensively used as immunoadsorbents for the isolation of antibodies. Proteins can be attached to insoluble polymers with retention of their biological activities by a large variety of methods (Silman & Katchalski, 1966; Campbell & Weliky, 1967; Crook, 1968; Kay, 1968). The number of potential methods reflects the fact that no one technique satisfies all the requirements in every instance, but it is apparent that certain methods are more widely applicable than others [e.g. bromoacetylcellulose (Robbins, Haimovich & Sela, 1967); Sepharose activated with cyanogen bromide (Axén, Porath & Ernback, 1967)]. It is, though, still necessary to screen a number of methods to determine which is most suitable for a particular purpose.

The present investigation arose from the need for an insoluble preparation of sperm-whale myoglobin that would be suitable for the isolation of antibodies to the *C*-terminal antigenic determinant of myoglobin. It was considered essential that the preparation should possess a high capacity for antibody and be suitable for use in a column, and also that the myoglobin should be attached to the insoluble support covalently but not through its tyrosine side chains, since the *C*-terminal tyrosine residue is probably an essential part of the *C*-terminal antigenic determinant (Atassi, 1968; Crumpton, Law & Strong, 1970). A water-insoluble derivative of sperm-whale myoglobin had been prepared previously by using an ethylene–maleic anhydride co-polymer (Givas, Centeno, Manning & Sehon, 1968). The procedure adopted for the preparation of the immunoadsorbent did not, however, appear entirely satisfactory in that the co-polymer was dissolved in anhydrous dioxan (Centeno & Sehon, 1966), whereas it has been reported that the co-polymer is insoluble in this solvent [Monsanto Technical Bulletin 1-261 (EX); Levin, Pecht, Goldstein & Katchalski, 1964]. The present paper compares the properties of immunoadsorbents prepared by covalently linking sperm-whale myoglobin to aminoethylcellulose, bromoacetylcellulose, CM-cellulose, an ethylene–maleic anhydride co-polymer and Sepharose 4B.

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MATERIALS AND METHODS

Materials. Aminoethylcellulose (AE11; batch no. 138) was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K., and bromoacetylcellulose (lot no. JC 269) was from Miles-Yeda Ltd., Rehovoth, Israel. CM-cellulose hydrazide (Enzite, batch H1) was purchased from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks., U.K., and the ethylene–maleic anhydride co-polymer (EMA-31; lot no. D-2615) was kindly given by Monsanto Europe, Brussels 5, Belgium. Sepharose 4B was from Pharmacia (G.B.) Ltd., London W.13, U.K. 4,7-Diphenyl-1,10-phenanthroline, ferric ammonium sulphate (AnalaR) and propionic acid were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.; the extinction (E_{1cm}) at 280nm of a molar solution of the propionic acid was 0.01. Cyanogen bromide was purchased from Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A., and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Sperm-whale metmyoglobin and apomyoglobin were prepared as described by Crumpton & Wilkinson (1965). Human albumin [10% (w/v) solution in 0.88% NaCl–0.13% sodium octanoate]

was kindly given by Blood Products Laboratory, Lister Institute, Elstree, Herts., U.K. Glass-distilled water was used in all experiments.

Antisera. Normal rabbit serum was obtained by pooling samples of serum that had been collected from about 30 animals before immunization. Rabbits were immunized with metmyoglobin in Freund's adjuvant as described by Crumpton & Wilkinson (1965). The immunoglobulin fraction of serum was prepared by precipitation with 14%-saturated Na_2SO_4 (Kekwick, 1940) and dialysed against 75mM-sodium phosphate–75mM-NaCl, pH 7.2; more than 95% of the antibody to metmyoglobin was recovered. Antisera and solutions of immunoglobulin were preserved with NaN_3 (0.01%), stored at −20°C and clarified by centrifuging before use.

Preparation of immunoadsorbents. Metmyoglobin (2ml of 75mg/ml) was attached to freshly regenerated aminoethylcellulose (0.5g) by the method of Weliky, Brown & Dale (1969), by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate (200mg in 3ml of water); the slurry was stirred for 4 days at 2°C.

Bromoacetylcellulose–myoglobin was prepared by the method of Jagendorf, Patchornik & Sela (1963); 165mg of metmyoglobin was added to 1g of the polymer. Maximal adsorption of the myoglobin was achieved in 0.15M-sodium phosphate–citrate buffer, pH 4.0.

CM-cellulose azide was prepared from 0.1g of CM-cellulose hydrazide by the method of Mitz & Summaria (1961), by using 2% (v/v) HCl and 2% (w/v) $NaNO_2$. The washed CM-cellulose azide was resuspended in 2.5ml of water and was stirred for 1h at 2°C with 50mg of metmyoglobin in 1ml of 0.1M-boric acid–NaOH buffer, pH 8.7.

Metmyoglobin was bound to the ethylene–maleic anhydride co-polymer by a modification of the method described by Centeno & Sehon (1966). The co-polymer was heated at 110°C for 24h (Levin *et al.* 1964) and a 0.6% (w/v) solution of the heated material in anhydrous acetone was added dropwise with stirring to 66mg of metmyoglobin in 12ml of 0.1M-sodium phosphate–citrate buffer, pH 6.0, or 0.1M-$NaHCO_3$, pH 8.0, at 2°C until no further precipitate formed; about 4ml of the co-polymer solution was added.

Sepharose 4B (settled volume 5ml) was activated with cyanogen bromide (500mg in 10ml of water) as described by Cuatrecasas, Wilchek & Anfinsen (1968), and washed with 0.1M-$NaHCO_3$, pH 9.0, at 2°C. The washed activated Sepharose was resuspended in 5ml of 0.1M-$NaHCO_3$, pH 9.0, 140mg of metmyoglobin was added immediately and the mixture was stirred for 20h at 2°C.

Each preparation was washed extensively by centrifuging with 75mM-sodium phosphate buffer–75mM-NaCl, pH 7.2, containing 0.01% NaN_3 (phosphate buffer–saline) and then M-propionic acid, pH 2.5, until no further material absorbing at 410nm or 280nm was eluted. No 410nm-absorbing material was subsequently eluted from the washed bromoacetylcellulose–, CM-cellulose– and Sepharose–myoglobin preparations with 8M-urea in 10mM-sodium phosphate buffer–0.15M-NaCl, pH 7.0, or 2M-KI in 50mM-tris–HCl buffer, pH 9.0. The immunoadsorbents were suspended in phosphate buffer–saline and stored at 2°C.

Determination of the amount of bound myoglobin. The amount of myoglobin attached to the various polymers was determined by amino acid analysis and from the iron content. Amino acid analyses were performed by using an automatic analyser (Benson & Patterson, 1965). Samples of known weight (2–8mg) of the washed immunoadsorbents that had been dried to constant weight *in vacuo* at 100°C were hydrolysed with constant-boiling HCl (1–4ml) *in vacuo* at 110°C for 24h (Crumpton & Wilkinson, 1963). The amount of myoglobin was calculated from the average recovery of the stable amino acids (arginine, glutamic acid, glycine, alanine, leucine, phenylalanine and tyrosine) and the known amino acid composition of sperm-whale myoglobin (Edmundson & Hirs, 1962).

The amount of material used for determination of the iron content was adjusted so that the amount of iron was less than 12μg. Dried samples (2–40mg) of the immunoadsorbents were digested with H_2SO_4, HNO_3 and $HClO_4$ as described by Kimura & Suzuki (1967). Iron was estimated in duplicate by the method of Doeg & Ziegler (1962). A freshly prepared 20% (w/v) solution of ascorbic acid was used to reduce the iron before extraction with the 4,7-diphenyl-1,10-phenanthroline reagent (0.83mg/ml of 3-methylbutan-1-ol). Ferric ammonium sulphate was used as the standard, and the standard curve was linear within the range of 0–12μg of iron.

Release of haem. Haem was extracted from myoglobin that had been attached to the insoluble polymers by using the method described by Crumpton & Wilkinson (1965) for preparing apomyoglobin. Suspensions of the immunoadsorbents (10mg/ml of water) were added with vigorous stirring to 50vol. of acetone–HCl (2ml of M-HCl/l of acetone) at −10°C. The insoluble material was recovered by centrifuging, re-extracted once with acetone–HCl as before, washed several times with water and dried. The amount of haem remaining after extraction was calculated from the iron content. Measurement of the protein content by amino acid analysis, before and after extraction, revealed that no protein was released by acetone–HCl.

Capacity of immunoadsorbent. The capacities of the immunoadsorbents to bind antibody to metmyoglobin were determined by using a batch procedure. Successive amounts (0.05–0.20ml) of the immunoglobulin fraction of an antimetmyoglobin serum (5.8mg of precipitable antibody/ml) were mixed with amounts of the immunoadsorbents that contained the same amount of attached myoglobin (2.75mg). After each addition of immunoglobulin the mixtures were centrifuged and the supernatants were examined for antibody by double diffusion against metmyoglobin in agar gel (Kabat & Mayer, 1961). Addition of immunoglobulin was continued until antibody was just detected in the supernatant. The total amount of antibody added was taken as the capacity of the preparation.

Elution of adsorbed antibodies. Samples of the immunoadsorbents containing a known amount of adsorbed antibody (see above) were washed successively at 2°C by centrifuging with 1ml volumes of each of the following solvents until E_{280}^{1cm} of the supernatant was less than 0.05: 2M-KI–50mM-tris–HCl, pH 9.0; 8M-urea–10mM-sodium phosphate–0.15M-NaCl, pH 7.4; 50mM-glycine–10mM-HCl, pH 3.0; M-propionic acid, pH 2.5; 20mM-HCl–0.15M-NaCl, pH 1.8. The supernatants of each solvent were pooled, adjusted immediately to pH 7.2 by addition of 4M-HCl or 4M-NaOH dropwise with stirring at 2°C, concentrated to 1–2ml by pressure dialysis against

phosphate buffer–saline and clarified by centrifuging. Recovery of protein was determined from the extinction at 280nm of the concentrated solution and the amount of precipitable antibody was determined by the quantitative precipitin test. In each case M-propionic acid, pH2.5, and 20mM-HCl–0.15M-NaCl, pH1.8, gave the highest recovery of protein, but the former solvent gave higher recoveries of precipitable antibody. As a result M-propionic acid was used as the eluting solvent in all experiments.

Isolation of antibodies to myoglobin by using Sepharose–myoglobin. The immunoglobulin fraction of an anti-metmyoglobin serum was added to a column (5cm × 1cm) of Sepharose-myoglobin (4mg of myoglobin/ml settled volume) in phosphate buffer–saline at 2°C. The column was washed with phosphate buffer–saline at a flow rate of 28ml/h and the effluent was monitored at 280nm by using a Uvicord II spectrophotometer (LKB Instruments Ltd., South Croydon, Surrey, U.K.); washing was continued until the extinction at 280nm of the effluent was the same as that before the immunoglobulin was added. The column was then eluted immediately with M-propionic acid, pH2.5, until the extinction at 280nm of the eluate returned to the initial value. The fractions corresponding to each peak were pooled separately, the pH of the propionic acid peak was immediately adjusted to 7.2 at 2°C and both peaks were concentrated to 1–2ml by pressure dialysis against phosphate buffer–saline. Recoveries of protein and precipitable antibody were determined as described above.

Other methods. The quantitative precipitin test was carried out as described by Crumpton & Wilkinson (1965). Concentrations of protein solutions were determined by measurement of the extinction at 280nm. The specific extinction coefficient ($E^{1\%}_{1cm}$) for solutions of metmyoglobin in water and in 0.1M-NaOH was 18.0; the values for rabbit immunoglobulin at neutral pH and in 0.1M-NaOH were taken to be 13.5 and 14.0 respectively (Crumpton, 1966).

RESULTS

Properties of myoglobin immunoadsorbents

The properties of the immunoadsorbents are compared in Table 1. Columns (6cm × 1cm) of aminoethylcellulose–, CM-cellulose– and Sepharose–myoglobin gave satisfactory flow rates, whereas bromoacetylcellulose– and ethylene–maleic anhydride–myoglobin could be used in a batch procedure only. Elution of columns (3cm × 0.6cm) of CM-cellulose– and Sepharose–myoglobin with normal rabbit serum (1ml) caused a noticeable decrease in the brown colour of the attached metmyoglobin. It seemed likely that the ferrihaem of at least some of the metmyoglobin had been preferentially bound by the serum albumin (cf. haemoglobin with human albumin; Bunn & Jandl, 1968). This interpretation was confirmed by elution with human serum albumin (1ml of 10mg/ml); the absorption spectrum of the eluted protein possessed a band with a maximum absorption at 405nm and was similar to that of methaemalbumin (Rosenfeld & Surgenor, 1950).

Table 1. *Properties of myoglobin immunoadsorbents*

The amounts of myoglobin bound by the various water-insoluble polymers were calculated from (*A*) amino acid analyses and (*B*) the iron contents. The release of haem was determined from the iron contents of samples of the immunoadsorbents that had been exhaustively extracted with acetone–HCl. Amounts of the immunoadsorbents containing the same weight of myoglobin (2.75mg) were saturated with antibodies by the addition of successive amounts (0.05–0.20ml) of the immunoglobulin fraction of an anti-metmyoglobin serum containing 5.8mg of precipitable antibody/ml. Adsorbed antibodies were eluted with M-propionic acid, pH2.5. The recovery of antibody was determined from the extinction at 280nm of the propionic acid eluate and is expressed relative to the amount of adsorbed antibody. The results are the average of two experiments. Experimental details are given in the text.

	Suitable for use as a column (6cm × 1cm)	Myoglobin bound (μg/mg dry wt. of immunoadsorbent) (*A*)	(*B*)	Haem released (%)	Antibody adsorbed (mg of precipitable antibody/2.75mg of bound myoglobin)	Antibody recovered (%)
Sepharose 4B	Yes	175	173	97	15.4	77
CM-cellulose	Yes	19.8	15.3	98	1.7	55
Aminoethylcellose	Yes	12.0	1.4	—	2.0	58
Bromoacetylcellulose	No	91	116	38	0.5	109
Ethylene–maleic anhydride, pH6.0*	No	420	459	0	2.2	0
Ethylene–maleic anhydride, pH8.0*	No	370	462	0	6.7	0

* pH at which myoglobin was attached to the polymer.

In contrast, no absorption band was detected within the range 350–450nm after elution with the immunoglobulin fraction of normal rabbit serum.

Amount of myoglobin bound. The amount of myoglobin attached to the water-insoluble polymers is shown in Table 1. The ethylene–maleic anhydride co-polymer bound the largest amount of myoglobin and similar amounts were attached at pH6.0 and 8.0. In contrast aminoethylcellulose and CM-cellulose bound very little myoglobin, and intermediate amounts of myoglobin were attached to Sepharose 4B and bromoacetylcellulose.

Amounts of myoglobin determined by amino acid analysis and from the iron content were in reasonable agreement for all preparations except aminoethylcellulose–myoglobin. Amino acid analyses of 24h hydrolysates of the immunoadsorbents revealed that in each case the molar proportions of arginine, glutamic acid, glycine, alanine, leucine, phenylalanine and tyrosine were in fairly good agreement with those for myoglobin. In contrast the lysine value was about half of that determined for myoglobin, and in some cases the amount of histidine was also low, whereas the aspartic acid and valine contents were invariably higher than those of myoglobin. These results suggested that myoglobin was attached primarily through its lysine side chains to the insoluble supports, especially since the low recovery of lysine and, to a smaller extent, histidine was unlikely to be due to the presence of carbohydrate during hydrolysis (Dustin, Czajkowska, Moore & Bigwood, 1953). The apparently abnormally high recoveries of aspartic acid and valine were probably due to the presence of extraneous substances some of which may have been formed by the reaction of ninhydrin with carbohydrate decomposition products. Indeed, hydrolysates of the immunoadsorbents contained unidentified ninhydrin-positive material that was eluted from the 50cm column of the analyser immediately before aspartic acid (cf. Dustin *et al.* 1953).

Release of haem. No decrease was detected in the iron contents of the ethylene–maleic anhydride–myoglobin preparations after extraction with acetone–hydrochloric acid. It was concluded that the haem of the attached myoglobin had not been released. However, under the same conditions all of the haem was extracted from Sepharose– and CM-cellulose–myoglobin, whereas less than half of the haem of bromoacetylcellulose–myglobin was released (Table 1). Since, in all probability, the release of haem depends on a conformational change in the protein moiety (Crumpton, 1966), it was concluded that the conformation of myoglobin attached to the ethylene–maleic anhydride co-polymer was not altered by acetone–hydrochloric acid. This was probably due to a stabilization of the conformation by a relatively large number of covalent bonds between each myoglobin molecule and the insoluble support. If this view is correct then myoglobin was probably attached to Sepharose and CM-cellulose by a smaller number of cross-links.

Adsorption and elution of antibody. Amounts of the immunoadsorbents containing the same amount of myoglobin (2.75mg) differed markedly in their capacities to adsorb antibody (Table 1); for example, the capacity of the most efficient immunoadsorbent (Sepharose–myoglobin) was about 30-fold that of the least efficient (bromoacetylcellulose–myoglobin). If it is assumed that 1mol of myoglobin binds a maximum of 3mol of antibody (Fig. 1 of Crumpton & Wilkinson, 1965), then 2.75mg of myoglobin in solution would combine with 73mg of antibody, whereas 15.4mg of antibody was adsorbed by 2.75mg of myoglobin attached to Sepharose. It was concluded that the capacity of Sepharose–myoglobin was approx. 20% of the maximum theoretical capacity, and that a portion of the attached myoglobin was not available to interact with antibody. With the other immunoadsorbents a larger portion of the attached myoglobin was inaccessible to antibody.

Each of the above immunoadsorbents containing adsorbed antibody was eluted successively with M-propionic acid (1ml fractions) until the E_{280}^{1cm} of the eluate was less than 0.05; if no 280nm-absorbing material was eluted the samples were washed with five 1ml portions of M-propionic acid. The eluates from each immunoadsorbent were pooled, adjusted to pH7.2 and concentrated to 1–2ml. All of the concentrated eluates except those from the ethylene–maleic anhydride co-polymer preparations gave a line of precipitation by double diffusion against myoglobin in agar gel. If it is assumed that all the eluted 280nm-absorbing material is antibody then all of the antibody adsorbed by bromoacetylcellulose myoglobin was released, whereas it appeared that some of the antibodies adsorbed by Sepharose–, CM-cellulose– and aminoethylcellulose–myoglobin were not eluted (Table 1). No protein was detected in the eluates of either of the ethylene–maleic anhydride–myoglobin–antibody preparations; although the failure to release antibody was probably due to lack of dissociation of the antibody–antigen complex, it seemed possible that it was related to the very marked decrease in volume (about sevenfold) of these preparations on treatment with propionic acid and that it may have been due to the occlusion of free antibody.

Samples of the immunoadsorbents were also treated with the immunoglobulin fraction of normal rabbit serum. The samples were washed exhaustively with phosphate buffer–saline and were then eluted with five 1ml portions of M-propionic

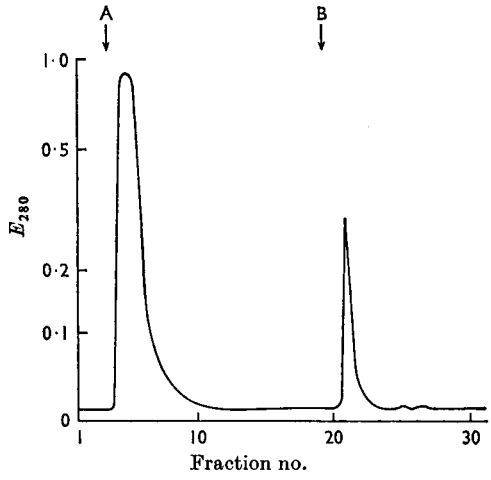

Fig. 1. Uvicord spectrophotometer trace of the extinction at 280nm of the effluent of a column (5cm × 1cm) of Sepharose–myoglobin (4mg of myoglobin/ml settled volume) equilibrated at 2°C with phosphate buffer–saline, pH7.2. The immunoglobulin fraction of an antiserum to metmyoglobin (1.5ml containing 94mg of non-specific immunoglobulin and 16.5mg of precipitable antibody) was added at arrow A and the column was washed with phosphate buffer–saline to arrow B, when the eluent was changed to M-propionic acid, pH2.5. The flow rate was 28ml/h, and 5ml fractions were collected.

acid. The pooled, neutralized and concentrated eluates contained less than 0.1% of the protein initially added. It was concluded that non-specific immunoglobulin was not adsorbed under the conditions at which antibody to myoglobin was bound.

Separation of antibodies to myoglobin

The results shown in Table 1 suggested that Sepharose–myoglobin was superior as an immunoadsorbent to the other preparations. As a result it was examined in greater detail. The separation of antibodies to metmyoglobin from non-specific immunoglobulin by using a column of Sepharose–myoglobin is illustrated in Fig. 1. A portion of the immunoglobulin added to the column was adsorbed and was subsequently eluted with propionic acid. In contrast no 280nm-absorbing material was detected in the propionic acid eluate when the immunoglobulin fraction of normal rabbit serum was added to the column. The amounts of precipitate formed by the various fractions with metmyoglobin and apomyoglobin are compared in Fig. 2. The results revealed that 77% of the added protein was not adsorbed by Sepharose–myoglobin, and that the unadsorbed protein gave no detectable precipitate with metmyoglobin. The adsorbed protein that was eluted with propionic acid (16.3mg; 98% of the antibody added to the column) contained approx. 14.1mg of precipitable antibody (87% of the eluted protein). Although the reaction of the eluted antibody with metmyoglobin and apomyoglobin was similar to that of the immunoglobulin fraction of the antiserum, the more bell-shaped precipitin curves (Fig. 2) suggested that the eluted antibodies were more heterogeneous than those originally present.

Similar results to those described above have been obtained with the immunoglobulin fractions of eight different antisera with different preparations of Sepharose–myoglobin. Metmyoglobin precipitated 60–90% of the protein eluted by propionic acid, and the recovery of antibody, relative to that added to the column, varied from 60 to 100%; in seven of the eight experiments the recovery was greater than 72%. The same column of Sepharose–myoglobin has been successively used, after re-equilibration with phosphate buffer–saline, on six occasions without any detectable decrease in capacity or alteration in specificity. Since the antibody-binding capacity of Sepharose–myoglobin was not decreased by re-use, recoveries of antibody of less than 100% were probably primarily due to the denaturation of eluted immunoglobulin rather than to incomplete dissociation of the antigen–antibody complex. Indeed, in some cases a white precipitate of denatured protein formed during the concentration of the neutralized propionic acid eluate, and lower recoveries of antibody were obtained if the propionic acid eluate was not neutralized immediately.

DISCUSSION

The myoglobin immunoadsorbents were assessed on the basis of the following criteria: suitability for column procedures, the conformation of the attached myoglobin, the nature of the amino acid residue cross-linked to the insoluble support, the capacity to adsorb antibody and the recovery of antibody. Although columns (6cm × 1cm) of bromoacetylcellulose– and ethylene–maleic anhydride–myoglobin were unsatisfactory, columns with satisfactory flow rates could probably have been obtained by mixing the immunoadsorbent with Sephadex G-25 (cf. Givas *et al.* 1968). Myoglobin attached to aminoethylcellulose contained negligible amounts of iron, whereas the other immunoadsorbents possessed approx. 1g-atom of Fe^{3+}/mol of protein. It appeared likely that the conformation of myoglobin attached to aminoethylcellulose had been altered during the coupling procedure so that the haem group was no longer bound by the protein moiety. Further, it was concluded that if myoglobin attached to Sepharose,

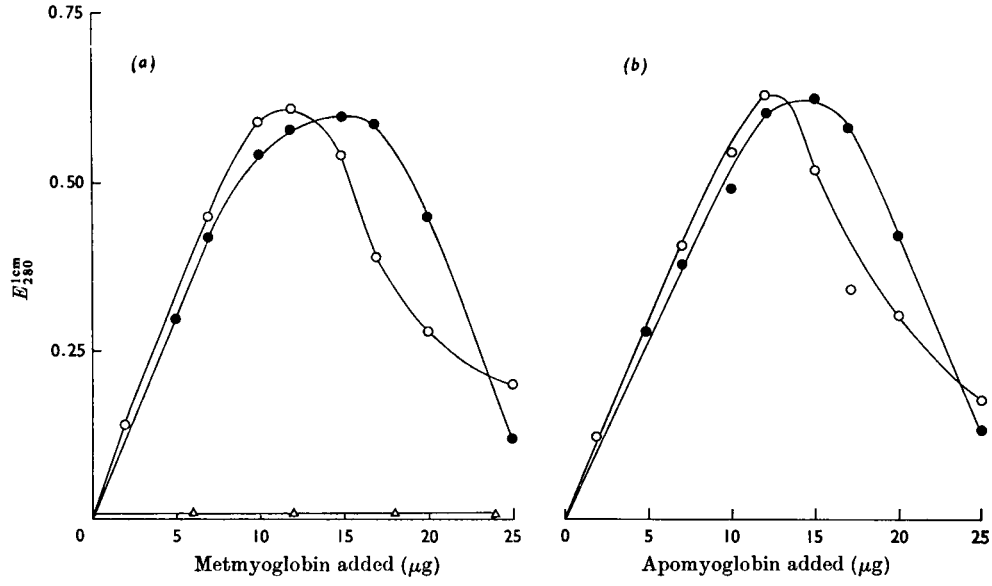

Fig. 2. Precipitin curves of (*a*) metmyoglobin and (*b*) apomyoglobin with the immunoglobulin fraction of the anti-metmyoglobin serum (○), the unadsorbed protein that was eluted from Sepharose–myoglobin with phosphate buffer–saline (△) and the adsorbed protein eluted with propionic acid (●). Increasing amounts of metmyoglobin or apomyoglobin were incubated for 1 h at 37°C and then at 2°C overnight with 20 μl of the initial immunoglobulin fraction (73 mg of protein/ml), 20 μl of the unadsorbed fraction (85 mg of protein/ml; total volume of fraction, 1.0 ml) or 100 μl of the adsorbed fraction (14.9 mg of protein/ml; total volume of fraction 1.1 ml); the volume in each tube was adjusted to 0.7 ml with 0.9% NaCl. The washed precipitates were dissolved in 0.5 ml of 0.1 M-NaOH and the extinction was measured at 280 nm in a 1 cm cell.

CM-cellulose, bromoacetylcellulose and ethylene–maleic anhydride co-polymer had undergone a conformational change then this had not resulted in a loss of haem. On the other hand, since the ferri-haem of CM-cellulose– and Sepharose–myoglobin was partially depleted by normal rabbit serum and human albumin, whereas no depletion was observed from solutions of metmyoglobin, it would appear that the conformation of at least some of the attached myoglobin was not identical with that of the native protein. Although the results of the amino acid analyses could not be interpreted unequivocally, the molar proportions of lysine and tyrosine relative to the stable amino acids suggested that myoglobin was attached to each support primarily through the lysine residues and that the tyrosine residues were not involved in cross-linkage. If it is assumed that the amount of haem released from the myoglobin immunoadsorbents by acetone–hydrochloric acid reflects the degree of cross-linkage, then ethylene–maleic anhydride– and bromoacetylcellulose–myoglobin were more extensively cross-linked than CM-cellulose– and Sepharose–myoglobin. In this case myoglobin attached to the former polymers was probably less accessible to antibody than myoglobin bound to CM-cellulose and Sepharose. The capacities of the immunoadsorbents to adsorb antibody appeared to depend on the accessibility of myoglobin rather than the amount of bound myoglobin. Sepharose–myoglobin possessed the highest capacity to adsorb antibody and the results indicated that at least 20% of the attached myoglobin was available to interact with antibody. The recovery of adsorbed antibody varied considerably for the different immunoadsorbents, but was highest for the bromoacetylcellulose and Sepharose preparations. Although no antibody was eluted from the ethylene–maleic anhydride–myoglobin preparations, antibodies to myoglobin have been isolated previously by using this immunoadsorbent (Givas *et al.* 1968). The reason for this disagreement is not known, but it may be related to the different methods used to prepare the immunoadsorbent and to elute the adsorbed antibodies. None of the immunoadsorbents adsorbed non-specific immunoglobulin under the conditions at which specific antibody was bound.

The above results suggested that Sepharose–myoglobin was the most efficient immunoadsorbent

for the isolation of antibodies to myoglobin. This view was supported by the results of more extensive investigations using column procedures. The overall yield of antibody was consistently greater than 72% of the antibody added to the column, and between 60 and 90% of the eluted protein was precipitable antibody; these values compare favourably with those reported for antibodies isolated by other techniques (e.g. Robbins *et al.* 1967). No evidence was obtained that any of the antigenic determinants of myoglobin had been masked during the coupling procedure. The immunoadsorbent was stable for at least 6 months at 2°C and has been re-used six times without any apparent decrease in specificity or antibody-binding capacity. It was concluded that Sepharose–myoglobin was eminently suitable for the fractionation of antibodies to myoglobin; a preliminary report of the isolation of antibodies to the *C*-terminal antigenic site of myoglobin by using this immunoadsorbent has appeared (Boegman & Crumpton, 1970). Further, it seems likely that the results of the present comparison are generally applicable to the fields of immunoadsorbents and enzymes attached to insoluble supports and that Sepharose will prove superior to other polymers as the insoluble support (cf. Kay & Lilly, 1970).

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