Antibodies elicited in rabbits against horse cytochrome c cross-react in varying degree with the cytochromes c of other species. Radioimmunoassay experiments are described in which 23% of the immunoglobulin shown to bind to horse cytochrome c fails to react with beef cytochrome c, and 14% of that bound by beef cytochrome c does not react with rabbit cytochrome c.

Studies of hybrid cytochromes, prepared by a reconstitution procedure involving the cleavage and re-formation of peptide bond 65-66, show that these differences result from single amino acid replacements. Beef cytochrome c differs in binding capacity from horse cytochrome c as a result of the substitution of a glycine for a lysine residue in position 60, and the difference between rabbit cytochrome c and beef cytochrome c reflects the substitution of a valine for a proline residue in position 44. Reconstituted horse cytochrome c and reconstituted beef cytochrome c have binding capacities indistinguishable from those of the parent proteins. The presence of a homoserine rather than a methionine residue in position 65 results, in the case of the reconstituted horse molecule, in a slightly lower affinity for the antibody population directed against the lysine-60 region. A corresponding difference is not observed in the case of beef cytochrome c and its reconstitution product, which do not bind the population in question.

The immune response to protein antigens involves recognition processes keyed to a limited number of residues. Attempts to identify these groups are at an early stage in the case of events at the cellular level, but have long constituted an important theme in studies of antibody binding. Such studies can be pursued most effectively with small proteins for which detailed structural information is available (see Refs. 1 to 3). These include the cytochromes c, which offer the special advantage that the amino acid sequences of preparations from numerous species have been determined. Through comparisons of the cross-reactivities displayed by these preparations, several antigenically significant regions have been explored (4-7).

Studies founded on comparisons of homologous molecules of natural occurrence are, of course, subject to significant restrictions. The information that can be obtained is dependent on the nature and location of the sequence differences available. Only the variable residues are subject to examination, and the approach works best when molecules differing at one or at most a few positions are compared. It is clear that, for systematic investigations, techniques permitting the formation of a series of more selectively varied structures must be brought into play. At the present time, some of the more promising of these involve the use of a reconstitution procedure that has been developed (8-10). Fig. 1 illustrates this process as it has been applied to cytochrome c from horse heart.

It has been shown that other cytochromes c with a methionine residue in position 65 can be split and reconstituted in a comparable manner, and that hybrid molecules can be formed by joining the heme peptide of the protein of one species to the non-heme peptide of another (11). In related experiments, it also has been demonstrated that the reconstitution process affords a suitable basis for the preparation of semisynthetic analogs of the cytochromes c (12). Both procedures provide opportunities for the generation of structures required for the immunochemochemical studies indicated. The results reported here illustrate the use of hybrids of the inter-species type.

**MATERIALS AND METHODS**

Cytochromes c—Cytochrome c from horse heart, beef heart, and rabbit heart was purchased from Sigma Chemical Co. (types III, V, and XV, respectively). Donkey heart cytochrome c was prepared by the procedure of Margoliash and Walasek (13). Beef heart cytochrome c dimer was a gift from Dr. L. Smith of this department. Reconstituted and hybrid cytochromes were prepared as described by Corradin and Harbury (8-11). Formic acid-treated horse cytochrome c was obtained by performance of the steps of the cyanogen bromide cleavage procedure (8), but with the cyanogen bromide omitted. All cytochromes were purified by chromatography on Amberlite CG-50 (13) or carboxymethylcellulose (Whatman CM52), followed by gel filtration through Sephadex G-50 equilibrated with PBS. In certain instances, further purification was effected by isoelectric focusing. All preparations displayed full electron transfer activity (14-16).

Other Proteins—Lactoperoxidase and BSA (crystallized) were obtained from Sigma Chemical Co. Goat anti-rabbit IgG serum was a gift from Dr. J. W. Patrick of the Salk Institute for Biological Studies, and sheep anti-rabbit IgG serum was a gift from Dr. M. Reichlin of the State University of New York at Buffalo.

Preparation of 125I-Cytochrome c (Horse)—Cytochrome c from horse heart was labeled with 125I in a manner similar to that described by Morrison et al. (17). NaI (10 mCi) was added to a solution of pH 7.0 containing 23 mM cytochrome c and 2 mM EDTA in 2 mM sodium phosphate buffer (4.3 ml). Following the addition of lactoperoxidase (12 mg in 25 µl), the reaction was initiated by the introduction of 20 µl of 0.03% hydrogen peroxide. The solution was permitted to stand for 30 min at 4°C.

The abbreviations used are: PBS, phosphate-buffered saline solution; 0.15 M sodium chloride and 0.002 M boric acid in 0.02 M sodium phosphate buffer, pH 7.0; BSA, bovine serum albumin; BrHo, rabbit anti-cytochrome c (horse); IgG, immunoglobulin G.

1 G. S. McLain and L. Smith, unpublished experiments.
at 23-25°C for 30 min and then subjected to the addition of a further 20 μl of hydrogen peroxide. After another 30 min, 5 μl of a more concentrated lactoperoxidase solution (25 μg/μl) and 10 μl of hydrogen peroxide were added to the reaction mixture. At the end of an hour, the latter was applied to a small column of Amberlite CG-50 equilibrated with a solution of pH 7.9 containing 20 mM sodium phosphate buffer and 0.1 M NaI. Following elution of the protein with 20 mM sodium phosphate buffer containing 0.3 M NaCl (pH 7.9), the cytochrome was reduced with sodium dithionite and passed through a column of Sephadex G-25 equilibrated with 20 mM phosphate buffer and 0.1 M NaI, pH 7.9. Samples were counted with a Hewlett-Packard Spectrophotometer (Hewlett-Packard model 3385). Two well resolved bands of radioactive material were obtained. Fractions corresponding to the first of these were pooled, oxidized with potassium ferricyanide, dialyzed against the gel filtration buffer, and passed through a column of Sephadex G-25 equilibrated with PBS. The labeled-cytochrome c so obtained was stored in the presence of BSA (1%). The specific activity of different preparations ranged from 3.65 to 4 mg of antigen in incomplete Freund's adjuvant (Difco). Nine rabbits were given approximately 20 μl of the antigenic preparation containing 0.1 ml of BSA. The surviving rabbit was then subjected to centrifugation at 12,000 g at 4°C for an additional 18 to 24 h and then subjected to centrifugation at 12,000 × g. The precipitate was washed with PBS and dissolved in 200 μl of 0.1 M NaOH. After 1 h at 25–25°C, samples of 100 μl were counted with a liquid scintillation spectrometer (Hewlett-Packard model 3085).

The antigen and antibody concentrations used in the competitive binding studies ensured that the antigen was present in large excess. From the initial slope of curves of the type shown in Fig. 2, the antigen was present in large excess. From the initial slope of curves of the type shown in Fig. 2, the antibody-binding properties of reconstituted horse cytochrome c were compared with those of the parent protein through quantitative precipitation experiments. Both studies yielded results that showed the two preparations to be antibody-binding properties of reconstituted horse cytochrome c were compared with those of the parent protein through quantitative precipitation and radioimmunoassay experiments. Both studies yielded results that showed the two preparations to be antigenically very similar (Figs. 4 and 5). However, it was apparent from the radioimmunoassay experiments that, to a small but significant extent, the reconstituted molecule competed less effectively with 125I-cytochrome c for the binding of Rho antibodies than did the naturally occurring protein. To deter-

FIG. 1. Cleavage and reconstitution of cytochrome c from horse heart. The reassembly occurs in two stages. In the first of these, a 1:1 complex is formed (Reaction 2). In stage 2, the amino group of residue 66 reacts with the homoserine lactone residue in position 65 to re-establish a molecule with an intact peptide chain (Reaction 3).
Antigenic Determinants of Cytochrome c

Donkey and Beef Cytochromes c—Donkey cytochrome c has a primary structure identical to that of horse cytochrome c except for the presence of a serine rather than a threonine residue in sequence position 47. The beef protein also contains a serine residue in position 47, but differs from the horse sequence in a total of three positions. The additional replacements occur at positions 60 and 89, where the beef molecule contains glycine residues and the horse protein contains a lysine residue and a threonine residue, respectively.

Fig. 3. Radioimmunoassay of horse cytochrome c. A, [125I]-cytochrome c/total cytochrome c: (a) 1.0; (b) 0.8; (c) 0.6; (d) 0.4; (e) 0.2; (f) 0.1; (g) 0.05. B, results of four experiments with three preparations of [125I]-cytochrome c.

Fig. 4. Quantitative immunoprecipitation of horse cytochrome c (●—●) and reconstituted horse cytochrome c (■—■).

mine whether this difference was a consequence of structural alterations other than the substitution of a homoserine for a methionine residue at position 65, competitive radioimmunoassays were conducted with horse cytochrome c treated with formic acid under the conditions used in the cyanogen bromide cleavage procedure. As shown in Fig. 5A, the results were indistinguishable from those obtained with the parent preparation. These findings suggest that the difference in antibody binding observed with the reconstituted material has its basis in the amino acid difference at position 65. This interpretation, it will be shown, is consistent with the results of other experiments to be described.

Reciprocal graphs of the radioimmunoassay data indicate that, at high ratios of competitor to [125I]-cytochrome c, the effect of the reconstituted cytochrome approaches that obtained with the natural molecule (Fig. 5B). The two preparations thus possess the same number of binding sites, and the difference in the degree of occupancy of these sites that is seen at lower concentrations reflects a difference in affinity rather than a difference in capacity. The ratio of the apparent association constants, estimated from the competitor concentrations at 50% inhibition, is 1.2.

Studies of antibody binding to these cytochromes yielded the data summarized in Fig. 6. The sequence difference between the horse and donkey proteins had a minor effect on the affinity and no effect on the binding capacity displayed by the antigen. In contrast, the difference between the horse and beef molecules affected the binding capacity very markedly. From the intercepts at the ordinate (Fig. 6B), it was found that 23% of the RbHo immunoglobulin bound by horse cytochrome c was not subject to binding by beef cytochrome c.

Horse-Beef and Beef-Horse Cytochromes c—The data for donkey cytochrome c indicate that the difference in the binding capacities of the horse and beef proteins is not attributable to the amino acid substitution in position 47. The basis is thus to be sought in the replacements at positions 60 and 89. To examine the effects of these amino acid differences individually, a sequence is needed which differs from that of horse or beef cytochrome c in terms of one of the substitutions only. The naturally occurring cytochromes of known sequence fail to meet this requirement, but this limitation can be overcome through the study of hybrid molecules (Table I).

For more information, please consult the full text document.
strate that the difference in the binding capacities of the parent proteins is a consequence of the amino acid substitution in position 60.

The three-dimensional structure of the cytochromes c places residue 60 within a short distance of residue 65 (23, 24). An amino acid substitution at position 65 could easily affect the binding of the fraction of RcHo immunoglobulin for which lysine-60 serves as a determinant. This almost certainly is the basis for the binding difference displayed by horse cytochrome c and its reconstitution product. Since beef cytochrome c does not bind RcHo immunoglobulin in the residue-60 region, it would be expected that a similar effect would not be observed in the case of this protein and its reconstitution product. The results given in Fig. 7 bear out this expectation. As can be seen, the binding properties of the reconstituted beef preparation were found to correspond closely to those of the parent molecule.

**Rabbit and Rabbit-Beef Cytochromes c**—The amino acid sequence of rabbit cytochrome c differs from that of beef at positions 44, 62, 89, and 92 (Table II). Comparison of the binding of RcHo immunoglobulin by the two proteins (Fig. 8) showed that 14% of the antibodies observed to bind to beef cytochrome c fail to bind to rabbit cytochrome c. In view of the findings concerning residues 60 and 65, this difference in binding capacity cannot be a consequence of the substitution at position 62.

The remaining substitutions, in position 44 and positions 89 and 92, occur in regions located diametrically across the molecule from one another. To ascertain whether the observed difference in binding is a reflection of the sequence difference in one of these regions or both, the properties of the hybrid rabbit-[1-65]-beef[66-104] molecule were examined (Fig. 8). The binding capacity was found to correspond to that of the rabbit rather than the beef protein. This observation establishes that the fraction of RcHo immunoglobulin that binds to beef but not to rabbit cytochrome c is keyed to proline residue 44. The occurrence of a valine residue in this position eliminates the binding.

**DISCUSSION**

In the radioimmunoassay experiments that have been described, 23% of the RcHo immunoglobulin found to bind to horse cytochrome c failed to bind to beef cytochrome c. Similarly, 14% of the RcHo immunoglobulin found to bind to beef cytochrome c did not react with rabbit cytochrome c. Through the study of hybrid molecules, it has been shown that each of these differences results from a single amino acid replacement. Beef cytochrome c differs in binding capacity from horse cytochrome c as a result of the substitution of a glycine for a lysine residue in sequence position 60, and rabbit cytochrome c has a lower binding capacity than beef cyto-

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**TABLE I**

Sequence differences used to identify an antigenic determinant present in horse cytochrome c but lacking in beef cytochrome c

<table>
<thead>
<tr>
<th>Cytochrome c</th>
<th>47</th>
<th>60</th>
<th>89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>Thr</td>
<td>Lys</td>
<td>Thr</td>
</tr>
<tr>
<td>Donkey</td>
<td>Ser</td>
<td>Lys</td>
<td>Thr</td>
</tr>
<tr>
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<td>Gly</td>
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<td>Thr</td>
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<td>Gly</td>
</tr>
<tr>
<td>Beef-horse</td>
<td>Ser</td>
<td>Gly</td>
<td>Thr</td>
</tr>
</tbody>
</table>

**FIG. 7.** Radioimmunoassay of horse cytochrome c (●—●), reconstituted horse cytochrome c (○—○), beef cytochrome c (▲—▲), reconstituted beef cytochrome c (□—□), horse-beef cytochrome c (▲—▲), and beef-horse cytochrome c (△—△).

**FIG. 8.** Radioimmunoassay of horse cytochrome c (●—●), beef cytochrome c (○—○), rabbit cytochrome c (□—□), and rabbit-beef cytochrome c (△—△).
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As a consequence of the presence of a valine rather than a proline residue in position 44. Collectively, the sites of which these residues are a part account for the binding of one-third of the antibodies observed to react with the horse protein.

With substitutions as radical as these, it is not surprising that antibody binding to the sites involved is completely suppressed. Other replacements, such as the substitution of a serine for a threonine residue at position 47 (donkey cytochrome c) or the substitution of a homoserine for a methionine residue at position 65 (reconstituted horse cytochrome c), resulted in changes in affinity but did not alter the binding capacity of the antigen.

A detailed characterization of any of the sites will require information concerning (a) the effects of stepwise variation of a series of residues in the regions of interest and (b) the results of structural changes of more than one kind at a given position. In part, the desired structures can be generated through the formation of hybrid molecules such as those used in the present experiments. Other changes in structure can be introduced by use of the reconstitution procedure in conjunction with standard chemical and enzymatic modification techniques. The most generally applicable approach, however, would involve the formation of products in which modified and unmodified heme peptides are joined to peptides prepared synthetically (12).

Such molecules incorporating modified and/or synthetic sequences may prove to be especially useful in studies of the binding sites for RCHo immunoglobin that remain to be identified. Preliminary observations suggest that the location of at least one of these may be difficult to establish through the examination of products consisting of natural sequence segments only. Direct evidence of the type presented for the lysine-60 and proline-44 regions should, on the other hand, be readily obtainable by the comparison of structures embodying other modifications.

REFERENCES


