Seven populations of site-specific antibodies were isolated from each of three sera of rabbits immunized against glutaraldehyde-polymerized horse cytochrome c. The antibodies were separated using an immunoadsorption scheme which employed the following cytochromes c: horse, beef, guanaco, rabbit, mouse testicular, pigeon, and the cyanogen-bromide cleaved fragment of the rabbit protein containing residues 1 to 65. The monovalent, antigen-binding fragments of the antibodies (Fab') gave 1:1 stoichiometries with native horse cytochrome c containing glutamic acid at position 44. One of these is specific for proline at that position, while the other antibody population also binds to cytochrome c containing glutamic acid at position 44. The remaining antibody population binds in the region of the lysine residue at position 60.

Each of the seven site-specific antibody populations binds effectively to any cytochrome c having a suitable amino acid sequence in the antigenic determinant regardless of any residue differences from the immunogen outside of that area. It was also demonstrated that these seven antibody populations represent the totality of the antibodies elicited in rabbits against horse cytochrome c, since the immunoadsorbants bound all the antibodies specific for the native protein. Furthermore, the rabbit antisera contained no other antibody population that could bind to the conformationally disturbed, cyanogen bromide-cleaved fragment of horse cytochrome c containing residues 1 to 65, making it appear that there were no antibodies elicited against a "processed" form of cytochrome c.

Antigenic determinants on protein molecules were early classified into two groups: sequential and conformational (1, 2). Molecules having sequential determinants can be enzymically cleaved without significant loss of antigenic activity, so long as the cleavage does not occur between amino acid residues within an antigenic determinant. Silk fibroin and bacterial flagellin are two such proteins (3, 4). Conformational determinants, on the other hand, require the proper spatial conformation of the polypeptide to ensure antibody binding. In such cases, even when peptide fragments of the molecule corresponding to the antigenic determinants bind antibodies, they do so with markedly decreased affinity as compared to the native molecule. Since most proteins have highly ordered conformations, their antigenic determinants are usually conformational in nature. Antigenic determinants are usually located on those parts of the polypeptide chain that are exposed in solution. For sets of globular proteins which have identical polypeptide backbone conformations, such as the eukaryotic cytochromes c, antigenic determinants which depend on differences in residue side chains have been termed epitopes by Jerne (5) and more recently topographic determinants by Urbanski and Margoliash (6, 7). Antibodies against such determinants, although requiring the native conformation of the antigen for binding, appear nonetheless to be directed against amino acid sequence differences between the host's and immunizing proteins.

A general approach used to identify antigenic determinants on a protein is to compare the immunologic cross-reactivities of a number of derivatives of the immunogen. Because homologous proteins from different species quite often have very similar amino acid sequences, they each represent a series of naturally occurring protein derivatives. The antigenic cross-reactivities of a number of homologous protein families have now been investigated including myoglobin (8), hemoglobin (9, 10), ribonuclease (11), ferredoxin (12), lysozyme (13), insulin (14), and cytochrome c (15).

The series of naturally occurring cytochromes c affords an excellent system to investigate the specificity of antibodies elicited against globular protein antigens (6, 7, 15). First, the amino acid sequences of over 85 different cytochromes c are now known, some of which differ by a single residue (16, 17). Secondly, the cytochrome c "fold" remains essentially the same for both eukaryotic and a variety of prokaryotic cytochromes c, even though they differ by over two-thirds of their amino acid sequences (13-21). Thus, the backbone conformations of eukaryotic cytochromes c that differ by relatively
Cytochrome c presents an interesting case in that from the rabbit protein at two amino acid positions, residues corresponding antibody or did so with decreased affinity. Guanaco protein at either position failed to bind the corresponding protein.

Thus, for example, guanaco cytochrome c differs from the rabbit protein to two amino acid positions, residues 62 and 89, the same two residues which were found to be immunodominant. Indeed, cytochromes c differing from the guanaco protein at either position failed to bind the corresponding antibody or did so with decreased affinity.

Antigenic sites on four cytochromes c have so far been identified (Table I). There is a clear correlation between the sites of antibody binding and the positions of the amino acid sequence differences between the immunizing and host’s cytochrome c. Thus, for example, guanaco cytochrome c differs from the rabbit protein at two amino acid positions, residues 62 and 89, the same two residues which were found to be immunodominant. Indeed, cytochromes c differing from the guanaco protein at either position failed to bind the corresponding antibody or did so with decreased affinity.

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Characterization of Antigen: The molecular weight of the specifically adsorbed antibodies was determined by gel filtration of a 1.0-mL solution of the protein and suitable standards on a column (1.5 x 60 cm) of Sephadex G-100 (Pharmacia). The heavy chain class of the antibody was determined by immunoellectrophoresis employing precast POL-E-FILM (Pfizer). Electrophoresis of the antibodies was for 45 min at 9 V/cm. Goat anti-rabbit γ and μ chains antiserum (Miles) was added to the buffer for blocking the background.

Preparation of Fab—Antibody solutions were dialyzed against BBS and concentrated to 5 mL using a 50-mL Amicon pressure filtration apparatus employing a PM-30 membrane. Final concentration to a volume of 0.5 mL was obtained using a Minicon concentrator (model B13, Amicon). An equal volume of 300 nm sodium acetate buffer, pH 4.0, was added to the antibody solution, followed by pepin (Walthington) (2% by weight relative to IgG). The antibodies were digested for 8 h at 37°C and the products separated on Sephadex G-100 (Pharmacia) in BBS. The Fab's were pooled, reduced with dithiothreitol and alkylated with iodoacetamide (1 and 4 mg, respectively, for each 10 mg of IgG). The Fab's were separated from the Fab fragments on a column (1.5 x 60 cm) of Sephadex G-25. All manipulations of antibodies were performed at 4°C.

Fluorescence Quenching Measurements—The assay was performed using the excitation wavelength of 285 nm and the fluorescence was determined at 335 nm (28) employing a Farrand manual spectrophotometer. Aliquots of 10-µL of a cytochrome c solution in BBS and 1 mL of the stock solution of the protein (1 mg/mL) were added from a 10-µL Hamilton syringe to the Fab solution (2 nmol/mL). As a control, cytochrome c was added to nonspecific IgG and the fluorescence determined. The proportion of active Fab in each preparation was determined by adsorption on insolubilized cytochrome c and was routinely 80 to 90% of the functional IgG from which the material was prepared. The concentration of antibody was determined spectrophotometrically assuming εz348 = 13,500 (29). Estimates of antibody binding constants were obtained from the fluorescence quenching data as described by Eisen and McGUIgan (30).

Competitive Binding Studies—A modified Farr assay (31) was employed. Horse cytochrome c was labeled with carrier free 125I (Amersham) by the chloramine-T (Eastman) procedure of Hunter and Greenwood (32). The experiments were performed as previously described (7). The amount of radioactivity precipitated at 50% saturation with ammonium sulfate was determined with the Packard automatic scintillation spectrometer.

Chemical Modification of Cytochrome c—The CDNP-residue 60 horse cytochrome c was prepared by the method of Brautigan et al. (33). This derivative was shown to be pure from peptide maps of tryptic and chymotryptic digests and to have maintained the native cytochrome c conformation by proton magnetic resonance spectroscopy (34). Horse and rabbit cytochromes c were chemically fragmented with sodium periodate and analyzed as described (35).

The abbreviations used are: CFA, complete Freund's adjuvant; Fab', monovalent antigen binding fragment containing the light chain and amino-terminal portion of the heavy chain; BBS, 8.2 mM borate buffer, pH 8.6, containing 130 mM sodium chloride; IgG, immunoglobulin G; CDNP, 4-carboxy-3,6-dinitrophenyloxysuccinimido; 125I, heavy chain class of the antibody was determined by immunoellectrophoresis employing precast POL-E-FILM (Pfizer). Electrophoresis of the antibodies was performed at 4°C and the products separated on Sephadex G-100 (Pharmacia) in BBS. The Fab's were pooled, reduced with dithiothreitol and alkylated with iodoacetamide (1 and 4 mg, respectively, for each 10 mg of IgG). The Fab's were separated from the Fab fragments on a column (1.5 x 60 cm) of Sephadex G-25. All manipulations of antibodies were performed at 4°C.
Rabbit Antibodies Directed Against Horse Cytochrome c

Comparison of the amino acid sequences of selected cytochromes c

The single letter code for amino acid residues is used. Only the residue differences from rabbit cytochrome c are shown. References to the amino acid sequences are provided in parentheses immediately after the species. The numbers of residue positions are given vertically, for example, 5 referring to residue 25. The symbol, δ, refers to the deletion of residue 104 in tuna cytochrome c, and “a” at the NH2 terminus indicates that the NH2-terminal glycine is N-acetylated.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Rabbit (36)</th>
<th>Horse (37)</th>
<th>Donkey (38)</th>
<th>Beef (39)</th>
<th>Mouse c (40, 41)</th>
<th>Guanaco (49)</th>
<th>Dog (43)</th>
<th>Mouse c (40)</th>
<th>Pigeon (44)</th>
<th>Duck (44)</th>
<th>Human (45)</th>
<th>Tuna (46, 47)</th>
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<tbody>
<tr>
<td>Rabbit 1-65 peptide</td>
<td>sGDVEK</td>
<td>GKKIF</td>
<td>VQKCA</td>
<td>QCHTV</td>
<td>EKGGK</td>
<td>HKTP</td>
<td>NLHGL</td>
<td>FGRKT</td>
<td>GGAVG</td>
<td>FSYTD</td>
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<td>1 2 3 4 5 0</td>
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Table II

RESULTS

Characterization of Rabbit Anti-horse Cytochrome c Sera

The amino acid sequences of the cytochromes c used in these studies are given in Table II. The somatic mammalian proteins listed differ by as few as 1 amino acid residue from horse cytochrome c as in the case of the donkey protein, or by as many as 9 residues, as for human cytochrome c, out of a total of 104. Besides the mammalian proteins, other cytochromes c utilized include those of the duck, pigeon, and tuna.

Antibodies elicited in rabbits against polymerized horse cytochrome c were separated on the basis of specificity using a series of immunoadsorption steps. Following exhaustive adsorption on insolubilized horse cytochrome c, the residual antibody activity in the filtrate was estimated by measuring its antigen binding capacity, using 125I-labeled soluble horse cytochrome c and precipitation of antigen-antibody complexes with ammonium sulfate of 50% saturation. It was found that all the antibodies directed against horse cytochrome c adsorb to the insolubilized protein. Hence, the immunoadsorption procedure developed accounts for the totality of the antibody populations. Competitive binding studies by the modified Farr technique indicated that the antigenic sites on the horse protein are probably located where horse cytochrome c differs from the rabbit protein, namely at residues 44, 47, 60, 62, 89, and 92 (data not shown). By assuming cross-reactivity between similar antigenic sites on different cytochromes c and through some trial and error, the isolation scheme given in Fig. 1 was developed.

Fig. 1. Scheme for the isolation of seven site-specific populations of rabbit anti-horse cytochrome c antibodies by immunoadsorption. The cytochromes c indicated in the figure and the 1-65 peptide were covalently attached to Sepharose 4B as given under “Experimental Procedures.” Horizontal arrows refer to non-adsorbed antibodies, vertical arrows to adsorbed antibodies. Antibodies not adsorbed at the first passage were reapplied as many times as needed to complete the adsorption. In those cases in which the pure antibody population was not adsorbed to the column which separated it in pure form, it was recovered by adsorption on insolubilized horse cytochrome c. The seven populations isolated are denoted in abbreviated form; for example, anti-89/92 (III) refers to anti-residues 89/92-subpopulation III.

Fig. 2. Separation of rabbit anti-horse cytochrome c populations directed against residue 44 (subpopulation II) and residues 89/92 (subpopulation IV). The antiserum used in this particular experiment was pooled from rabbits 2008 and 2012 and treated according to the scheme in Fig. 1. Panel A shows the elution from a column containing insolubilized 1-65 peptide of rabbit cytochrome c of the antibody which had previously adsorbed to pigeon cytochrome c. The open circles show the eluate of the first adsorption, the closed circles that of the second. Panel B shows the elution from insolubilized horse cytochrome c of the antibody that failed to adsorb to the 1-65 peptide. The open circles show the eluate of the first adsorption, the closed circles that of the second.

REFERENCE

12708
TABLE II—continued

<table>
<thead>
<tr>
<th>ANANK</th>
<th>GITWG</th>
<th>EDTLM</th>
<th>EYLEN</th>
<th>PKKYI</th>
<th>PGTKM</th>
<th>IFAGI</th>
<th>KKKDE</th>
<th>RADIJ</th>
<th>AYLKK</th>
<th>ATNE</th>
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Fig. 3. Isolation of rabbit anti-horse cytochrome c anti-residue 60 antibody. The antiserum from rabbit 2010 was first adsorbed on insolubilized beef cytochrome c and eluted as shown in Panel A. The open circles show the eluate of the first adsorption, the closed circles that of the fourth. The unadsorbed antibody was adsorbed on insolubilized horse cytochrome c and eluted as shown in Panel B. The open circles show the eluate of the first adsorption, the closed circles that of the second.

Table III

<table>
<thead>
<tr>
<th>Antibody population</th>
<th>Stoichiometric ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera from rabbits</td>
<td>2007</td>
</tr>
<tr>
<td>Total antibodies</td>
<td>3.0, 2.9</td>
</tr>
<tr>
<td>Anti-residue 44-subpopulation I</td>
<td>0.9</td>
</tr>
<tr>
<td>Anti-residue 44-subpopulation II</td>
<td>N.D.</td>
</tr>
<tr>
<td>Anti-residue 60</td>
<td>N.D.</td>
</tr>
<tr>
<td>Anti-residue 89/92-subpopulation I</td>
<td>0.9</td>
</tr>
<tr>
<td>Anti-residue 89/92-subpopulation II</td>
<td>1.1</td>
</tr>
<tr>
<td>Anti-residue 89/92-subpopulation III</td>
<td>0.9</td>
</tr>
<tr>
<td>Anti-residue 89/92-subpopulation IV</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

In an attempt to observe whether antibodies directed against some form of cytochrome c resulting from possible processing of the protein by the immune system, the cyanogen bromide cleaved 1-65 peptide of horse cytochrome c was used. This peptide comprises two-thirds of the amino acid residues of the native protein and exists in a relatively random coil conformation (48). Thus, should antibodies be elicited against denatured cytochrome c or an enzymically cleaved peptide (likely candidates as processed materials) the 1-65 peptide would bind many of these antibodies. This turned out not to be the case. Indeed, following immunoadsorption of pooled antisera elicited against either monomeric or polymerized horse cytochrome c, the filtrates were passed on immunoadsorption columns containing horse cytochrome c 1-65 peptide.
There were no antibodies that bound the 1-65 peptide but did not bind native horse cytochrome c.

**Isolation and Identification of Site-specific Antibodies**

Following the isolation scheme given in Fig. 1, seven different antibody populations were isolated. Four of them were found to be directed against the region of residues 89/92, two against the region of residue 44, and one against the region of residue 60. Each of the three rabbit antisera examined could be fractionated into these seven populations. However, the proportions of these populations were not the same for each serum, as listed in Table IV. The greatest variation was in the anti-residue 60 antibody which comprised 40% of the total antibody in one case (rabbit 2010) and less than 20% of the antibody in the other two antisera.

The identification of the antigenic determinants corresponding to the seven populations of antibodies isolated is discussed below and in the miniprint supplement immediately following the article. The identification relies heavily on the amino acid sequences of the various cytochromes c employed, listed in Table II. It should be noted that binding of antibodies to a column of insolubilized cytochrome c can be complete under the conditions employed even if the binding affinity to the cytochrome c in solution is relatively low. Thus, the anti-residue 44-subpopulation II antibodies could be totally adsorbed in three filtrations through insolubilized 1-65 peptide (Fig. 2A). However, in fluorescence quenching experiments (Fig. 4) a 1-65 peptide molar excess of 67 times that of antibody sites gave only about 80% of the maximal quenching obtained with native horse cytochrome c. The latter reached 95% of the maximal quenching at a 1:1 molar ratio with antibody sites. The corresponding estimated affinity constants of this antibody population are about 10^4 M^-1 with the 1-65 peptide and 10^5 M^-1 or higher with native horse cytochrome c. In the same way, differences in fluorescence quenching observed with different cytochromes c and each of the purified antibody populations probably represent differences in affinity (see under “Discussion”), and it is these differences in affinity which are used below to identify the corresponding antigenic determinants.

Because of the complexity and detail of the antibody specificity determinations, only two of the antibody populations will be discussed in this section. These antibodies were isolated as shown on the right-hand side of the horizontal arm of the purification scheme (Fig. 1). The rationale detailed below is representative of that used in the identification of the specificity of the remaining five antibody populations which are

**Table IV**

Relative amounts of antibody directed against each antigenic determinant on horse cytochrome c

<table>
<thead>
<tr>
<th>Antibody population anti-residue:</th>
<th>Sera from rabbits</th>
<th>2007</th>
<th>2008/2012</th>
<th>2010</th>
</tr>
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<tbody>
<tr>
<td>60</td>
<td></td>
<td>19</td>
<td>19</td>
<td>40</td>
</tr>
<tr>
<td>89/92-Subpopulation I</td>
<td></td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>89/92-Subpopulation II</td>
<td></td>
<td>9</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>89/92-Subpopulation III</td>
<td></td>
<td>9</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>89/92-Subpopulation IV</td>
<td></td>
<td>N.D.</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>44-Subpopulation I</td>
<td></td>
<td>9</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>44-Subpopulation II</td>
<td></td>
<td>N.D.</td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

* The antisera of both animals were pooled.

* These two fractions together represent 48% of the total antibody, but the amount of each of the two site specific antibodies was not determined (N.D.).

**Fig. 4.** Fluorescence quenching titrations of rabbit anti-horse cytochrome c Fab' from anti-residue 44-subpopulation II obtained from rabbits 2008 and 2012 with horse cytochrome c (O), 1-65 peptide (O), and 1-80 peptide (C) from horse cytochrome c. The apparent binding constants are 10^4 M^-1, 10^5 M^-1, and 10^6 M^-1, respectively.

**Fig. 5.** Fluorescence quenching titrations of rabbit anti-horse cytochrome c Fab' prepared from anti-residues 89/92-subpopulation II. The antibodies were isolated from the serum of rabbit 2007.

Discussed in the miniprint supplement.

**Anti-residues 89/92-Subpopulation II**—Some of the antibodies in the rabbit anti-horse cytochrome c sera that did not adsorb to the insolubilized rabbit or guanaco proteins were adsorbed by beef cytochrome c. This antibody population was identified as anti-residues 89/92-subpopulation II. The corresponding antigen binding fragments gave a 1:1 stoichiometry with horse cytochrome c (Fig. 5) and are directed against one of the two regions of the molecule where guanaco and beef cytochromes c differ. These differences are at residue 44 where beef cytochrome c has proline and the guanaco protein carries valine, and 92 where the corresponding residues are glutamic acid and alanine (Table II). Although the dog protein is identical to beef cytochrome c in the regions of residues 1-84, these antibodies were not adsorbed on the insolubilized dog protein. Clearly then, they are not directed against the region of residue 44 but rather against the region of residue 92.

Fluorescence quenching titrations of the Fab' derived from this population with several cytochromes c are shown in Fig. 5. Glutamic acid at position 92 is a requirement for antibody binding. This is demonstrated by the fact that the beef and mouse c proteins (glutamic acid at position 92) quenched the
fluorescence, while the mouse c, did not react at all, even though it is identical throughout this region with the beef protein, except for an alanine at position 92. Residue 89 is also involved in the antibody binding since horse cytochrome c, which differs from the beef and mouse c, proteins at that position, bound the antibody with higher affinity. The difference in binding between beef cytochrome c and mouse c, is probably due to the isoleucine residue at position 96 in the mouse c, protein, close to residue 92 in the COOH-terminal helix.

Anti-residue 60—Insolubilized beef cytochrome c adsorbs most of the antibodies directed against horse cytochrome c, ranging from 60 to 80% depending on the antiserum. The antibody not adsorbed by the beef protein was subsequently adsorbed by horse cytochrome c (see Fig. 3). The corresponding Fab' showed a 1:1 stoichiometry with the horse protein and failed to bind to a modified form of horse cytochrome c, the CDNP-lysine 60 derivative (Fig. 6). Since this modified protein has been shown to have the native conformation (34), the effect on the binding of the antibody must be due to a structural change in the vicinity of residue 60. Because none of the other cytochromes c, with the exception of the donkey protein, bind to this antibody, it was impossible to determine the contribution of nearby residues to this antigenic determinant. One cannot rule out the possibility that the amino acid at position 62, which also represents a difference between the horse and rabbit proteins, is involved in the binding of the antibody to the region of residue 60. In any case it is quite clear that residue 62 is not immunodominant, since beef cytochrome c failed to adsorb the antibody directed against this region and both the horse and beef proteins carry glutamic acid at position 62, where the rabbit has aspartic acid.

**DISCUSSION**

The approach used in the identification of antibody binding sites on cytochrome c involves three steps. First, competitive binding assays of the whole serum antibodies using a variety of cytochromes c show the degree to which the different proteins cross-react (7, 15). From this information one can develop a scheme for the isolation of site-specific antibody populations employing a series of insolubilized cytochromes c (7). Finally, the populations are identified either by direct or competitive binding assays. The fluorescence quenching technique (29) used to measure binding directly also yields the stoichiometry of the reaction so that one can determine the presence of one or more than one single site-specific antibody population.

Each of the rabbit anti-horse cytochrome c antibodies isolated discriminates between different cytochromes c in antibody fluorescence quenching. Presumably this discrimination results from differences in affinity of the antibody for the various antigens. This interpretation assumes that at sufficiently high concentrations of antigen, fluorescence quenching would in each case have reached the maximum it readily attains with an antigen having a high affinity to the antibody. An alternative explanation is that, over and above affinity differences, the orientation of antigen and antibody in the complex changes as the structure of the antigenic determinant changes. Thus, at points on the fluorescence quenching curves which appear to be reaching a maximum, nearly all the antibody is bound, but it is the differences in orientation which account for the decreased level of fluorescence quenching. Regardless of which interpretation is correct, differences in fluorescence quenching can be used to identify antigenic determinants, as described above.

Although there are only three regions on horse cytochrome c where rabbit anti-horse cytochrome c antibodies bind, seven antibody populations were separated on the basis of differences in specificity. Within these seven populations there surely are further subpopulations having subtle specificity differences which were not detected using the isolation scheme shown in Fig. 1. Nonetheless, it was possible to distinguish differences in specificity among antibodies directed against even a single site. Fig. 7 shows two views of the backbone spatial structure of cytochrome c. The residues which differ between horse and rabbit cytochromes c, as well as those found to be immunodominant for these antibodies, are indicated.

One of the antigenic sites on horse cytochrome c involves two amino acid sequence substitutions, at residues 89 and 92, and thus constitutes a complex site. Four antibody populations directed against this area of the molecule have been isolated. One of the antibody binding sites (anti-residues 89/92 subpopulation I) is centered around residue 89, a second (anti-residues 89/92 subpopulation II) around residue 92, while a third population (anti-residues 89/92-subpopulation III) appears to have a binding site centered around both residues 89 and 92. The site of binding of this population, and perhaps also the others, encompasses residues 88 and 96 as well as the more centrally located and dominant residues 89 and 92. All of these are on the COOH-terminal helix of cytochrome c and within a distance of 12 Å from each other (Fig. 7). The fourth population directed against this region of the molecule (anti-residues 89/92-subpopulation IV) is less specific in its binding than the other three, and, in fact, adsorbs to every cytochrome c examined.

At the bottom right of the molecule (Fig. 7) two antibody populations directed against the region of residue 44 were isolated. These antibodies differ in their degree of specificity. Thus, anti-residue 44-subpopulation I directed against a proline/valine substitution does not bind to cytochromes c containing glutamic acid at that position, while anti-residue 44-subpopulation II binds to such proteins. The observation that horse and donkey cytochromes c (differing only at position 47) bind identically to these antibodies makes it appear that residue 47 is not involved in the site of binding.

The last of the rabbit anti-horse cytochrome c antibody populations was directed against the region of residue 60 near the center of the backbone surface of the molecule (Fig. 7). The specificity of this population is less completely defined than the others simply because horse and donkey cytochromes c were the only two proteins that bound the antibody. None of
FIG. 7. Diagrams of the backbone conformation of cytochrome c indicating the positions at which horse cytochrome c differs from the rabbit protein (heavily outlined circles at α carbons) and the immunodominant residues for rabbit anti-horse cytochrome c antibodies (arrows). A view of the molecule from the front surface containing the exposed heme edge is given in Panel A and a view from the right-hand side in Panel B. The heme is the square structure seen edge-on in the front view and from the side in the side view (darkened atoms). The larger numbered circles are the α carbons. Taken from an electron density map of tuna ferricytochrome c at 2 Å resolution (20).

The other known cytochromes c has lysine at position 60, the immunodominant residue. Thus, it is impossible to examine the contributions of nearby residues to the binding of the antibody using this approach without preparing proteins that are chemically modified in that area.

Despite the observation that antibodies elicited against heterologous cytochromes c bind in regions of the molecule where amino acid sequence differences occur between the immunizing the host proteins, in several cases it is not possible to conclude that the dominant residues of the antigenic determinant are actually the amino acid sequence differences. Thus, of the rabbit anti-horse cytochrome c antibodies, anti-residue 44-subpopulation II and anti-residues 89/92-subpopulation IV bind to every cytochrome c examined. Although these residues are immunoactive in that they affect the binding of the antibodies, because no amino acid residue modification of the horse protein is known to prevent the binding of these two antibody populations, the immunodominant residues in the corresponding antigenic determinants cannot be identified.

It is important to emphasize that these seven rabbit anti-horse cytochrome c antibody populations are the totality of the antibodies made in our experiments. Indeed, after removing from the antisera those antibodies that react with the insolubilized native protein, no antibodies directed against soluble native cytochrome c remained. Furthermore, since no antibodies were observed that bound to the random-coil, NH2-terminal heme peptide fragment of the protein comprising residues 1 to 65, other than those reacting with native cytochrome c, it appears that the antibody producing mechanisms when presented with the native protein responds to cytochrome c in its native conformation. There are no antibodies produced to any form other than the native, structurally intact protein. These observations strongly militate against the possibility that antigen processing, if it occurs at all, involves cleavage of peptide bonds before the specificity of the antibodies that will be made is decided.

Although horse and rabbit cytochromes c differ at 6 residue positions (44, 47, 60, 62, 89, and 92) we have shown that only 4 of those residues are involved in antibody binding (residues 44, 60, 89, and 92). Thus, the conservative amino acid substitutions at positions 47 (threonine/serine) and 62 (glutamic/aspartic) appear not to elicit antibodies and may not even be part of antigenic determinants. This effect may be due to the presence of a more strongly immunogenic residue within the same region. For example, residue 62, where horse and guanaco cytochromes c both carry glutamic acid and there are no other amino acid sequence differences from the rabbit protein in that area of the guanaco protein, is in fact antigenic in rabbits immunized against the guanaco protein (7). Presumably then, the antibody response against lysyl residue 60 in the horse protein, where rabbit cytochrome c has glycine, precludes the response against the adjacent aspartic to glutamic acid change at residue 62.

Unlike the above situation, the contribution of residue 47 to the binding of the anti-residue 44-subpopulations I and II could be examined directly. Horse and donkey cytochromes c differ only at position 47 where the horse protein carries threonine and both donkey and rabbit cytochromes c have serine. Results of fluorescence quenching titrations and competitive binding assays indicate that the antibodies directed against horse cytochrome c in the region of residue 44 do not discriminate between the two substitutions at residue 47. A similar result using unfractionated rabbit anti-horse cytochrome c sera was obtained previously (49). Had residue 47 been contained in the antigenic determinant, one may have expected the assays to detect the difference between a threonyl and a seryl residue. Indeed, in one known case these residues have been distinguished by rabbit anti-human cytochrome c antibodies (49). Human cytochrome c has tyrosine...
and serine at positions 46 and 47, respectively, where the rabbit and donkey proteins carry phenylalanine and serine, while the horse protein has phenylalanine and threonine. The anti-human cytochrome c serum cited did differentiate between horse and donkey cytochromes c, which vary only at residue 47.

A large proportion of the rabbit anti-horse cytochrome c antibody (50%) is capable of binding rabbit cytochrome c. Because antibodies bind not only to the amino acid residue differences but also to other neighboring residues, quite often an antibody will bind to a protein that does not carry the amino acid sequence difference by virtue of which the antibody had presumably been elicited. Thus, for example, an antibody population directed against the proline residue at position 44 in horse cytochrome c (anti-residue 44-subpopulation I) cross-reacts, although more weakly, with guanaco (or rabbit) and mouse cytochromes c which carry valine and alanine at that position, respectively, but does not bind to pigeon cytochrome c which has glutamic acid at residue 44. The analogous antibody elicited in rabbits against guanaco cytochrome c also binds the mouse protein but with a decreased affinity (7). Similarly, in the region of residues 89 and 92 in horse cytochrome c, several antibodies directed against a threonyl and glutamyl residue at those positions in horse cytochrome c also bind the guanaco protein which carries a glycine and alanine at those positions, respectively. These side chains in guanaco cytochrome c are shorter than those in the horse protein so that the antibody can be accommodated. On the other hand, the analogous antibody elicited against guanaco cytochrome c fails to bind the horse protein (7). By investigating many antigenic determinants in this manner it will be possible to catalog immunologic cross-reactivities against the types of amino acid differences involved, eventually enabling one to explain the phenomenon of immunologic cross-reactivity in precise molecular terms.

While this manuscript was in the process of revision, two independent reports appeared in the literature which are consistent with the findings discussed above. Eng and Reichlin (50), using a series of immunoadsorption columns, isolated three populations of rabbit anti-horse cytochrome c antibodies directed against the regions of residues 44, 60, and 89/92. Berman (51) and Harbury (52), using unfractionated antisera and hybrid cytochrome c molecules (recombinants of the species, the peptides resulting from cyanogen bromide cleavage of cytochrome c), also showed that residues 44, 60, and 89 influence the antigenicity of horse cytochrome c.

The isolation of the site-specific antibody populations elicited in rabbits against the three antigenic determinants on horse cytochrome c has proved to be useful beyond its immunological implications. Such antibodies can serve as indicators of localized changes in the conformation of the protein (53) as probes of the functional interaction between cytochrome c and its electron-exchange partners (54) as described in the following paper (55).

**Acknowledgments**—We are grateful to Dr. D. L. Brautigan for providing the highly purified CDNP-lysine 60 modified horse cytochrome c preparation, to Dr. G. Goldberg and Dr. T. Wheat for the mouse testicular tissue from which mouse c was obtained, and to Dr. R. E. Dickerson for supplying the stereoscopic diagrams from which Fig. 7 was prepared.

**REFERENCES**

Rabbit Antibodies Directed Against Horse Cytochrome c

Additional references appear on p. 12716.
Rabbit Antibodies Directed Against Horse Cytochrome c

by R. Jaworski and E. Mengphasch

Further Details of the Identification of the Site-Specific Antibodies

The specificity of the two antibody populations was identified and described under Rabbit Anti-Horse 99/97 Subpopulation I and Anti-
Residues 44-62. The specificity of the remaining five site-specific antibody populations are discussed below.

Anti-Residues 89-92. Subpopulation II—Isoelectric rabbit cytochrome c

This requirement eliminates residues 44, 47, and 52 as possible sites. Finally the failure of the dog protein to bind the Fab, although the beef and dog cytochrome c are identical at position 89, again implicates the region or residues only as the antigenic determinant. It is possible that residues 95 in mouse c (lysine) inhibited the binding of this protein to this population. The only other difference between mouse c and the horse protein in this region is at residue 92, serine and threonine, respectively. This serine is likely to prevent the binding since beef cytochrome c with a glycine as at position 89 bound effectively. However, residues 95 and 96 also differ in this region only at the position.

Fluorescence quenching titrations of rabbit anti-horse cytochrome c Fab' from Anti-Residues 99/97 Subpopulation III (pooled from Rabbits 2007 and 2009).

Fig. 10. Fluorescence quenching titrations of rabbit anti-horse cytochrome c Fab' from Anti-Residues 99/97 Subpopulation III (pooled from Rabbits 2007 and 2009).

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12716 Rabbit Antibodies Directed Against Horse Cytochrome c

Fig. 11. Competitive binding assays for antibodies adsorbed to horse cytochrome c according to the separation scheme in Fig. 1. The purified antibodies were diluted with normal rabbit serum. For each antibody, the blue bar represents the highest absorbance after addition of duplicate samples containing the antigens (in the absence of Fab). The upper left symbol indicates the highest absorbance at 0.66 mg of Fab. The unlabeled horse cytochrome c was displaced with horse cytochrome c to give an antibody binding equivalence of 0.005 mg of Fab. The Fab' fragments of the two antibodies showed a stoichiometry with horse cytochrome c, and similar differences in binding between the cytochromes tested as was previously observed with the other antibodies directed against the region of Residues 44/92. These two antibody populations appear to be less specific in their binding than the other antibody populations directed against the same determinant. The Anti-Residues 89/92 Subpopulation I antibody bound with the highest affinity to horse cytochrome c followed by the beef protein and mouse (Fig. 11). These three cytochromes carry glutamic acid at position 92, so that, in confirmation of the results obtained with the modified Fab' assay (Fig. 11), these differences in binding must be due to changes at position 89. However, the glutamic acid at Residue 92 is also involved in the binding. Indeed, guanaco cytochrome c, although identical to the beef protein at Residue 92, binds the antibody with decreased affinity because of the aspartic acid at position 89. The rabbit protein is only slightly less effective than guanaco cytochrome c in binding the antibody, presumably because of the aspartic acid replacement for glutamic acid at position 89.

Fig. 12. Competitive binding assays for antibodies adsorbed to horse cytochrome c according to the separation scheme in Fig. 1. The purified antibodies were diluted with normal rabbit serum. For each antibody, the blue bar represents the highest absorbance after addition of duplicate samples containing the antigens (in the absence of Fab). The upper left symbol indicates the highest absorbance at 0.66 mg of Fab. The unlabeled horse cytochrome c was displaced with horse cytochrome c to give an antibody binding equivalence of 0.005 mg of Fab. The Fab' fragments of the two antibodies showed a stoichiometry with horse cytochrome c, and similar differences in binding between the cytochromes tested as was previously observed with the other antibodies directed against the region of Residues 44/92. These two antibody populations appear to be less specific in their binding than the other antibody populations directed against the same determinant. The Anti-Residues 89/92 Subpopulation I antibody bound with the highest affinity to horse cytochrome c followed by the beef protein and mouse (Fig. 11). These three cytochromes carry glutamic acid at position 92, so that, in confirmation of the results obtained with the modified Fab' assay (Fig. 11), these differences in binding must be due to changes at position 89. However, the glutamic acid at Residue 92 is also involved in the binding. Indeed, guanaco cytochrome c, although identical to the beef protein at Residue 92, binds the antibody with decreased affinity because of the aspartic acid at position 89. The rabbit protein is only slightly less effective than guanaco cytochrome c in binding the antibody, presumably because of the aspartic acid replacement for glutamic acid at position 89.
Topographic antigenic determinants on cytochrome c. Immunoabsorbent separation of the rabbit antibody populations directed against horse cytochrome.

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