STUDIES ON THE ANTIGENIC PROPERTIES
OF FERREDOXIN FROM
CLOSTRIDIUM PASTEURIANUM

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

In the Department
of
Microbiology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
25 March, 1970
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Date May 19, 1970
ABSTRACT

It was established that antibodies could be evoked in rabbits against ferredoxin purified from cultures of Clostridium pasteurianum, and against its performic acid oxidized derivative. The extent of cross-reaction was studied between the two antisera and four related antigens: native ferredoxin, iron-sulfide free ferredoxin, performic acid oxidized ferredoxin, and S-carboxymethylated ferredoxin. All combinations demonstrated cross reactivity by complement fixation, and in the case of oxidized ferredoxin antiserum, three preparations, native ferredoxin, iron sulfide free ferredoxin, and performic acid oxidized ferredoxin precipitated antibody.

The data obtained with these cross-reactivity studies indicated that the cysteine-containing regions of the ferredoxin molecule were not critically involved as antigenic determinants. The C-terminal region of the protein was considered for further study. This octapeptide was synthesized and tested for its ability to combine with antibody directed against both native ferredoxin and its performic acid oxidized derivative. The peptide exhibited specific binding to both antisera as demonstrated by inhibition of complement fixation and precipitation, and by equilibrium dialysis experiments.

It is suggested that C. pasteurianum ferredoxin is antigenic in rabbits, that cysteine residues are not involved in at least two of the antigenic regions of the protein, and that the C-terminal octapeptide is one of the antigenic determinants of this molecule.
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ACKNOWLEDGEMENTS

I would like to thank Dr. Julia Levy for her many suggestions, helpful criticism, and invaluable encouragement during the research and writing of this thesis. I also wish to thank Dr. H. Whitely for kindly supplying cultures of Clostridium pasteurianum and for her suggestions concerning the culturing of the organism. And I wish to extend my gratitude to Mrs. Barbara Mitchell and John Christensen for their cooperative contributions and to Mrs. Barbara Mitchell for drawing many of the figures used in this thesis. Finally, I extend thanks to Dr. Doug Kilburn and Dr. Tony Warren for their suggestions concerning the final draft.
INTRODUCTION AND LITERATURE REVIEW

Until recently the antigenic determinants of naturally occurring proteins have been determined for only a few proteins and within rather broad limits; certainly the smallest sequence of amino acids in a protein capable of binding with antibody directed against the whole antigen has been determined for only one protein, that of tobacco mosaic virus. Molecular studies on peptide antigens have been carried out using mainly synthetic homopolymers or heteropolymers of known composition, though not necessarily known sequence. Many reviews of this work have been published in the last five years (Kabat, 1966; 1968; Sela, 1966; Gill, Kunz, Freedman and Doty, 1963; and Proceedings of the Royal Society, Series B, 1966: Gabe, Richards and Sela). The work is extensive and most conclusions refer to general properties of the antigenic determinants such as charge, size, conformation, and chemical properties. These will be dealt with only insofar as they relate to the work discussed in this thesis.

In order to obtain useful information concerning the immune response to naturally occurring proteins, the sequence of peptides found to bind antibody directed against the whole molecule should be placed within the context of the entire sequence and structure of the protein. Lately, the amino acid sequences and conformations of many proteins have been determined, making such a study feasible.
The ferredoxin molecule of *C. pasteurianum* is especially suited to such a study. It is easily purified in suitable quantities; it is a small protein (MW 5500), with few aromatic residues (1 phenylalanine and 1 tyrosine); it has a single basic residue (lysine), making it extremely electronegative; and it contains eight cysteine residues, relatively evenly spaced in groups of four along its sequence, with no disulfide bridges; lessening the chance of conformational variables. Also, ferredoxins from several sources have been purified and their sequences established. They all demonstrate a high degree of sequence homology (Tsunoda and Yasunobu, 1968), and provide a naturally occurring series of antigens which can be studied for their cross-reacting properties, and ultimately for relationships of antigenic sites from different species. This thesis presents initial studies on determining the antigenic determinants of ferredoxin from *Clostridium pasteurianum*.

1. Determination of Antigenic Sequences

The principle method employed for the determination of the antigenic determinants of naturally occurring proteins involves the isolation of peptides from the parent molecule and the subsequent testing of their ability to bind with antibody produced against the whole molecule. Although a number of globular protein antigens, such as bovine serum albumin (Porter, 1957), human serum albumin
(LaPresle et al., 1957, 1960), diphtheria toxin (Raynaud et al., 1959) and thyroglobulin (Metzgar et al., 1962), yielded fragments by enzyme hydrolysis which were capable of combining with antibodies evoked by intact antigens, these fragments were of high molecular weight and appeared to have more than a single antigenic determinant (polyvalent) since they gave precipitates with homologous antisera.

Press and Porter (1962) isolated a chymotryptic peptide from human serum albumin which caused 50% inhibition of precipitation with the homologous system when tested at a molar concentration 1.5 times that of the intact antigen. This peptide did not precipitate anti-HSA serum by itself. The molecular weight of the fragment was estimated to be 7100 and no attempt was made to elucidate its chemical structure, aside from its amino acid composition, or to determine if a smaller portion of the peptide was capable of binding antibody.

An octapeptide of composition gly(gly₃ala₃) tyr which was isolated from silk fibroin by Cebra (1961) inhibited precipitation in the homologous system by 40 to 90%, depending on the serum used. Glycine, alanine, and tyrosine compose 45.9%, 26.4% and 5.6% respectively of the fibroin molecule (Florkin and Stotz, 1963). Removal of the C-terminal tyrosine with carboxy-peptidase caused a 50% decrease in the peptide's binding to anti-fibroin serum. No attempt was made to locate the peptide in the fibroin molecule.

Brown (1962) isolated active peptides from oxidized bovine
ribonuclease and found one peptide, encompassing residues 39 to 62, to be 50% as active as the intact antigen in inhibiting specific precipitation, while not precipitating antibody itself. No attempt was made to find the smallest peptide in this sequence capable of binding antibody directed against the whole molecule.

Four chymotryptic peptides, ranging in length from 7 to 19 residues, were shown by Crumpton and Wilkinson (1965) to inhibit precipitation between metmyoglobin (containing haem) and its homologous antiserum, while they did not precipitate antibody themselves (Figure 1). Atassi and Saplin (1968) isolated 5 tryptic peptides from sperm whale myoglobin which inhibited precipitation of the whole molecule by its homologous antiserum, while they had no precipitating activity themselves. These peptides are also shown in Figure 1.

It is interesting to note that four of these peptides occupy corner positions in the three-dimensional model of the protein, while the fifth is the C-terminal hexapeptide (Figure 2). They also found that cyanogen bromide cleaved the protein at the methionine residues at positions 55 and 131, producing three fragments: the first two fragments (1-55 and 56-131) formed immune precipitates, and each contained two of the five tryptic peptides found to be antigenic; the third fragment showed inhibitory activity only, and was found to contain the last of the active peptides. Together the three fragments produced by cyanogen bromide (CNBr) cleavage accounted for 100% of the precipitating capacity of the antisera. Atassi
Val^{1} - Trp^{7} - (Ala^{15} - Val^{17} - Arg^{31} - Phe^{33}) - Lys^{34}

Ser^{35} - Phe^{43} - V - Met^{55} - (Lys^{56} - Leu^{69})

Thr^{70} - Lys^{79} - Lys^{96} - His^{97} - Tyr^{103}

His^{19} - V - Met^{131} - Asn^{132}

- (Arg^{140} - *^{146}) - (Lys^{147} - Glu^{148} - *^{151} - Gly^{153})

Figure 1. Studies on antigenic sites of Sperm Whale Myoglobin.

() - enclose haptene chymotryptic peptides (Crumpton & Wilkinson, 1965).

[ ] - underline haptene tryptic peptides (Atassi & Saplin, 1968).

V - cleavage points of Cyanogen Bromide (Atassi, 1967).

* - nitrated Tyrosines (Atassi, 1968).
Figure 2. Tertiary structure of Sperm Whale Myoglobin (Atassi & Saplin, 1968).

The approximate position of reactive tryptic peptides are shown by marking on the main line.
and Saplin also found that cleavage of the N-terminal heptapeptide at tryptophan 7 left the molecule immunologically intact, in agreement with Crumpton and Wilkinson (1965) who found this peptide unreactive.

The results of the work involving separation of antigenic peptides from sperm whale myoglobin are summarized in Figure 1. Of the two areas of correspondence between the results of Crumpton and Wilkinson (1965) and those of Atassi and Saplin (1968), one of them, residues 17-31, is found in a corner piece between two helical regions, and thus could be more available to the antibody synthesizing machinery; the second area of correspondence involves the C-terminal residues, which, in the three-dimensional model, also appear readily available (Figure 2).

In each report the complete chymotryptic and tryptic digests gave no more than 25% inhibition, while the 3 fragments obtained by Atassi and Saplin with CNBr cleavage accounted for all the reactivity of the intact antigen. It is likely that some determinants were split by the proteolytic enzymes, or that isolated peptides underwent extensive configurational changes which prohibited proper alignment for binding to antibody.

Chemical modification of specific residues in myoglobin supports the data found using isolated peptides. Atassi (1967) found that oxidation of the two methionine residues with periodic acid had no effect on the immune reactivity of the whole molecule, indicating
that these two residues are not involved in antibody binding. Atassi (1968) also investigated the role of tyrosine in the antigenic reactivity. Nitration at Tyr$^{103}$ of the fragment 56-131 obtained by CNBr cleavage, did not reduce reactivity compared with the un-nitrated control. Nitration of tyrosines 146 and 151 in the fragment 132-153 caused complete loss of reactivity. As Figure 1 shows, Tyr$^{103}$ is not found in a reactive peptide, while tyrosines 146 and 151 are.

Antigenic peptides have been isolated from hen egg-white lysozyme which are capable of inhibiting homologous precipitation with the whole molecule. This protein, too, is an excellent subject for such a study, since its amino acid sequence and tertiary structure have been established (Canfield, 1956 and Blake, 1965). Shinka et al (1967) isolated four peptic peptides capable of inhibiting precipitation by 25% while not precipitating antibody alone. All four peptides were found to cover the sequence Glutamine$^{57}$ to Alanine$^{107}$ with different single peptide bond breaks at residues 83 and 84 in a loop formed by a disulfide bridge (Figure 3). One of these peptides (with a break at Leucine$^{83}$) was hydrolyzed further with trypsin, and one peptide, encompassing residues Tryptophan$^{65}$ and Lysine$^{96}$, was recovered which delayed the development of turbidity in the homologous precipitation reaction. No attempt was made to degrade the peptide further or to determine the effect of disrupting the disulfide bridges. Fujio et al (1968) isolated a peptic peptide encompassing
the N-terminal residues, Lysine\textsuperscript{1} to Asparagine\textsuperscript{27}, joined to the C-terminal residues, Alanine\textsuperscript{122} to Leucine\textsuperscript{129}, by a disulfide bridge at position 127, which he found to possess specific inhibiting power (Figure 3).

Atassi and Habeeb (1969) studied the effect of modifying Tyrosines 20 and 23 on the antigenicity of hen egg-white lysozyme. They found that nitration of these two residues decreased the ability of the molecule to precipitate homologous antiserum by 10 to 33% depending on the serum used. This inhibition is in fair agreement with that found by Atassi (1967) when sperm whale myoglobin was nitrated. Reduction of the nitrotyrosines to aminotyrosine brought about an almost complete recovery of the antigenic activity. On nitration of tyrosine ortho to the phenolic hydroxy group, the electron withdrawing nitro group increases the acidity of the phenolic hydroxy, while reduction causes a reversal of this effect (pK\(a\) Tyrosine = 10.1, pK\(a\) NO\(2\)-Tyr = 7.2, pK\(a\) NH\(2\)-Tyr = 10.0). The two tyrosines occur in the N-terminal portion of the peptide found by Fujio \textit{et al} (1969) to be antigenic.

In a study on reduced and carboxymethylated lysozyme, Gerwing and Thompson (1969) isolated a single tryptic peptide encompassing the sequence Asparagine\textsuperscript{74} to Lysine\textsuperscript{96} which gave a maximum inhibition of precipitation of 60%. This sequence falls within the peptide found by Shinka \textit{et al} (1967) to be antigenic. Removal of the three N-terminal amino acids of the peptide completely destroyed its inhibitory
Figure 3. Amino acid sequence of Hen Egg-White Lysozyme (Shinka et al, 1967).
activity, while a synthetic decapeptide covering the sequence Asparagine to Leucine was found to be inhibitory. Correlation between this work and that done by other workers on native lysozyme is of doubtful value considering the drastic changes in configuration caused by breaking the disulfide bridges (there is no cross-reaction between native lysozyme antiserum and CM-lysozyme). No attempt has been made as yet, to determine if a smaller sequence than the decapeptide is capable of binding with anti-CM-lysozyme antisera.

II. Importance of Configuration

The importance of configuration in antibody binding has been studied to a limited extent with respect to naturally occurring protein antigens. Spragg et al (1968) studied the effect of single amino acid substitutions in the nonapeptide bradykinin with antibody evoked against the peptide linked to a polylysine carrier. Alanine was substituted for different amino acids in the peptide, and the effect of the substitutions on antibody binding was tested. The results indicated that alterations in charge or hydrophobicity (Alanine for Arginine, Serine or Phenylalanine) had little effect on binding, while substitutions causing greater freedom or restriction on conformation (Alanine for Glycine or Proline) caused a great reduction in binding.

Work on globular proteins shows, generally, that methods which alter conformation, such as disruption of disulfide bridges, destroy
reactivity with antiserum evoked against the native protein. Brown (1959) showed that performic acid oxidized ribonuclease did not cross react with native ribonuclease antiserum, and similar results were observed by Thompson and Gerwing (1969) employing reduced and carboxymethylated hen egg-white lysozyme and antiserum directed against the native protein.

Merrigan et al. (1966) extended the work on the role of conformation in the antigenicity of ribonuclease, and found that denaturation by heat or urea completely destroyed the ability of RNase S (obtained by cleavage between residues 20 and 21 with the NH$_2$-terminal fragment attached to rest of the molecule by a disulfide bridge) and RNase S protein (RNase minus peptide 1-21) to form precipitates with antibodies directed against native RNase in agar gel. Under normal conditions these two derivatives produce reactions of partial identity, indicating that they retain some antigenic sites found in the native molecule, while they are incapable of binding all antiribonuclease antibody.

A similar study was carried out by Bonavidà et al. (1969) on hen egg-white lysozyme. They cleaved the two methionine residues in the molecule at positions 12 and 105, but the three peptides formed are held together by disulfide bridges, so the molecule remains intact. The cleaved molecule was found to inhibit the native system to a maximum of 70% and showed partial identity with native hen egg-white lysozyme antiserum in agar gel, indicating the loss of some antigenic determinants. Changes in conformation were
indicated by a red shift in the ultra-violet spectrum of the altered molecule, consistent with tryptophan being found in a more hydrophobic environment.

Arnon and Sela (1969) studied the dependence of antibody specificity on conformation using the "loop peptide" of hen egg-white lysozyme, which encompasses residues 64 to 83 of the protein. They isolated the peptide from a peptic digest of lysozyme and coupled it to a synthetic copolymer of alanine and lysine. Antibodies directed against this conjugate were raised in rabbits. Purified "loop" peptide was found to inhibit the conjugate precipitin system 75% and reduced and carboxymethylated "loop" was almost as effective; native lysozyme was also found to be an inhibitor of this system to the extent of 35%. The purified "loop" peptide was capable of inhibiting the lysozyme-anti-lysozyme system 5%, while the reduced and carboxymethylated "loop" was inactive. The anti-"loop" antibodies obtained with lysozyme on cross immunoeabsorption could discriminate between the peptide still containing a disulfide bridge and the same peptide after the bridge was opened, to a much greater extent than the anti-"loop" antibodies purified from sera directed against the synthetic polypeptide conjugate. It appeared to the authors that the anti-"loop" antibodies are directed against a conformation dependent determinant, the loop being a more rigid conformation within native lysozyme than when attached to the conjugate.

Finally, Crumpton and Small (1967) compared the conformation of two active peptides obtained from sperm whale myoglobin and their
effects on antibody binding. In solution, one of the peptides, Bj, contains 14 amino acid residues of which \( \frac{12}{14} \) are helical in the native molecule. The other, Cla, contains 13 amino acids, of which 8 in the middle are non-helical. Optical rotary dispersion studies indicated that both peptides were essentially non-helical in solution, yet both were found to bind with antibody evoked against the native molecule. The authors suggest that in solution the peptides possess a variety of conformations in continuous interchange and that antibodies react with those molecules whose conformations are the same as that of the corresponding region in the native protein; the optical rotary dispersion studies indicated that a small portion of the molecules of peptide Bj may indeed possess the helical conformation in aqueous solution although it could be no more than 5%. If this interpretation is correct, it is possible that the helical conformation of peptide Bj is stabilized by interaction with antibody.

III. Nature of the Antigen Determinant

The most detailed study to date on naturally occurring proteins has been carried out by Benjamini and co-workers on tobacco mosaic virus protein. They found a single tryptic peptide, containing 20 amino acids, to have specific haptenic activity (Benjamini et al, 1964, 1965). Removal of 5 amino acids from the NH\(_2\)-terminus of the peptide and 2 from the COOH-terminus, produced a tetradecapeptide which
was capable of binding anti-TMVP antibody, although binding was somewhat reduced compared to the eicosapeptide (Young, et al, 1966). Stewart et al (1966) synthesized the COOH-terminal decapeptide portion of the eicosapeptide and demonstrated that it possessed haptenic activity. Following this, a series of peptides of increasing length from the COOH-terminal end of the decapeptide was synthesized and tested for binding activity. It was found that anti-TMVP antibody bound the pentapeptide to the nonapeptide, while the di-, tri-, and tetrapeptides lacked significant activity. The sequence of amino acids in the pentapeptide was Leu-Asp-Ala-Thr-Arg.

The minimum size of 5 amino acids is in close agreement with the results of other studies. Arnon et al (1965), studying rabbit serum albumin conjugated with polylysine peptides of an average chain length of 5.5 residues, found that polylysine peptides showed inhibitory power in the homologous system which increased sharply up to penta-lysine and then levelled off rapidly up to the nonapeptide. Antibodies directed against the poly-D-glutamic acid capsule of Bacillus anthracis were shown to bind maximally with hexa-poly-D-glutamic acid in the case of two antisera, and with the pentapeptide in a third case (Goodman et al, 1968). Kabat (1960), studying antibodies against dextran, found that a hexasaccharide was the minimal antigen size capable of binding. The dimensions of hexa-glutamic acid are 36 x 10 x 6 Å and those of isomaltohexaose, 34 x 12 x 7 Å. The similarity of the size of the two determinants is
especially significant since they were obtained from very different biological substances.

Having isolated the smallest sequence of amino acids of the TMV protein eicosapeptide capable of binding antibody elicited against the whole protein, Benjamini et al (1968) investigated the properties of the peptide that were responsible for specific binding. They found that the coupling of octanoic acid to the NH$_2$-terminus of the COOH-terminal tri- and tetrapeptides caused them to exhibit specific hapten activity, while the octanoyl dipeptide was inactive, indicating the importance of the hydrophobic character of the NH$_2$-terminal amino acids. To study the relative importance of the 5 amino acids in specific binding, Young et al (1967) synthesized a number of analogues of the peptide. The importance of the NH$_2$-terminal hydrophobicity was established, since D- or L-alanine, isoleucine or tyrosine could be substituted for leucine with retention of activity. It appeared that the shape, rather than the negative charge of the aspartic acid, at position 4 from the COOH-terminus, was important since asparagine but not glutamic acid could be substituted with conservation of haptenic activity. A peptide of sequence Thr-Leu-Asp-Ala-Thr was inactive, while a peptide of sequence Asp-Ala-Thr-Arg-Arg was active. Threonine and arginine occur at the NH$_2$- and COOH-terminal ends of the active pentapeptide in the whole TMV protein; the arginine-arginine bond is split by trypsin during isolation of the eicosapeptide. These results suggest the importance of hydrophobic
interactions in the binding between antibody and antigen and also serve to emphasize that results obtained with isolated peptides obtained by enzymic hydrolysis must be interpreted with care, since antigenic sites may be fragmented by the action of the enzyme.

With a defined antigenic determinant, the workers were able to compare the specificity of antibodies obtained from different animals. While none of the antisera bound the COOH-terminal di-, tri- or tetrapeptides, sera produced by some rabbits bound the pentapeptide while others required the hexa- or heptapeptide for demonstrable binding. The amount of pentapeptide bound by antibodies produced by a given rabbit did not necessarily correlate with the amount of decapeptide bound by antibodies produced by the same rabbit. However, it was found that the octanoyl-pentapeptide was bound by all sera tested, including those that did not bind the unsubstituted pentapeptide. The effect was specific for anti-TMVP serum since anti-lysozyme serum showed no binding. The authors have suggested that the octanoyl group enhances a possible hydrophobic interaction between the antigenic area and the antibodies (Benjamini et al., 1969) which stabilized the complex, while specificity is determined by the pentapeptide region.
MATERIALS AND METHODS

I. Cultures

(a) Organism

The culture of *Clostridium pasteurianum* used in this study was kindly supplied by Dr. H. Whitely, Department of Microbiology, University of Washington, Seattle. It was the same strain used by Tanaka et al. (1966) to obtain ferredoxin for the sequencing of the protein.

(b) Stock Cultures

Stock cultures were maintained in the following medium (Whitely, H., personal communication, 1969): peptone, 1%; beef extract, 1%; yeast extract, 0.5%; sodium acetate, 0.5%; Cysteine-HCl, 0.5%; soluble starch, 0.1%; and agar, 0.2%. The medium was dispensed in 20 ml quantities to 19 x 150 mm tubes, closed with metal caps and autoclaved for 15 min at 15 lb pressure. Just prior to inoculation the medium was immersed in boiling water for 15 min and rapidly cooled. A 10% inoculum was used for all culturing procedures and incubation was carried out at 30 C for 18 hr. The cultures were stored at 4 C and subcultured every 3 months.
(c) Batch Cultures

Organisms from which ferredoxin was purified were grown in 30 litre quantities in 8 gallon stainless steel milk cans. Cultures were grown in the synthetic medium of Lovenberg (1963), with \((\text{NH}_4)_2\text{SO}_4\) as nitrogen source. The 30 litres of medium were prepared by dissolving the constituents in 27 litres of tap water in the milk can. Sterilization was accomplished by bubbling steam through the medium for 1 hr, by way of a 1.5 cm diameter stainless steel tube attached to the lid of the can and which extended to within 10 cm of the bottom of the container. At the end of the sterilization time, the medium, which had increased to 30 litres in volume, was cooled rapidly to 30 C in running tap water. The inoculum for batch cultures was prepared by serially inoculating from a stock culture to 250 ml of the maintenance medium and then to 3 litres of the synthetic medium which was used as inoculum for the batch culture. The batch culture was incubated for approximately 18 hr at 30 C.

(d) Harvesting

The cells from the 33 litre batch cultures were harvested by continuous flow centrifugation using an 8-tube Szent-Gyorgi-Blum continuous flow apparatus (Ivan Sorvall Co.) in a Serval RC2 refrigerated centrifuge. Cells were sedimented at 30,000 x g at 0 C, with a flow rate of 150-200 ml/min. To decrease
foaming during harvesting, 5.0 litre portions of the medium were degassed under vacuum for 15 min and 0.2 ml of tributyl citrate was added as a defoaming agent, prior to centrifugation. The harvested cells were placed in a beaker and held at -10 °C overnight.

II. Purification of Ferredoxin

Ferredoxin was purified from the frozen and thawed cell paste according to the method of Mortensen (1964) as modified for a cell paste by Lovenberg and Sobel (1965). The thawed cells were suspended in 600 ml of distilled water at 4 °C and stirred with a magnetic stirrer for 1 hr. Then an equal volume of acetone at 4 °C was added and stirring was continued for 5 min at 4 °C. The acetone slurry was clarified by centrifugation at 20,000 x g for 30 min at 4 °C. The sedimented material was discarded and the ferredoxin was further purified from the supernatant by column chromatography.

DEAE cellulose (Biorad Co.) was repeatedly clarified to remove fines and poured into a 25 mm diameter column to a height of 50 mm. The column was equilibrated with 1.0 M phosphate buffer at pH 6.5 and then washed with 500 ml of distilled water. The 50% acetone cell extract was run through the column with a flow rate of 10 ml/min. The yellow column effluent was discarded. The column was then washed with 250 ml of distilled water to remove any acetone, and then with
0.15 M Tris-HCl buffer pH 8.0 until the effluent had an absorbance at 260 μm, 1.0 cm light path of a less than 0.10. The ferredoxin was eluted with 0.65 M NaCl buffered with 0.15 M Tris-HCl pH 8.0 in as small a volume as possible and was desalted by dialysis against 0.015 M Tris-HCl buffer pH 7.3 for 6 hr at 4 °C.

Final purification was achieved by ammonium sulphate precipitation. Some contaminating material was removed from the desalted ferredoxin solution by addition of solid (NH₄)₂SO₄ to 60% saturation. Precipitation was allowed to occur at 4 °C for 6 hr and the precipitate was removed by centrifugation at 20,000 x g for 20 min. The ferredoxin was precipitated by increasing the (NH₄)₂SO₄ concentration to 90% saturation by addition of solid salt. Precipitation was allowed to occur overnight at 4 °C, and then the precipitate was recovered by centrifugation at 20,000 x g for 20 min. The reddish supernate was discarded. The precipitate was dissolved in a minimum of 0.015 M Tris-HCl buffer pH 7.3, and dialysed against 1 litre of the same buffer for 10 hr with stirring at 4 °C. The dialysate was changed frequently during this time. The 290/280 μm absorbance ratio of the desalted preparation was determined using a Beckman DBG spectrophotometer; and the ferredoxin was repeatedly precipitated with ammonium sulphate at 90% saturation until the ratio ceased to increase. Yields were calculated using the extinction coefficient at 390 μm of \( E_{1}^{1%} \) cm = 33.2 with 0.015 M Tris-HCl buffer as diluent, as described by Mortenson (1964).
III. Analytical Procedures

(a) Spectral Analysis

The absorption spectra from 200 μm to 450 μm of the purified preparations of ferredoxin were determined on a Unicam Recording Spectrophotometer Model SP 800 B with a Model SP 20 recorder, using a 1 cm light path, and 0.15 M Tris-HCl buffer as diluent.

(b) Amino Acid Analysis

All preparations of ferredoxin and its modified derivatives were checked for their amino acid composition. Samples were pipetted into glass ampoules and an equal volume of 12 N HCl was added. The ampoules were sealed under vacuum and hydrolysed for 18 hr at 110°C. The hydrolysates were washed three times and taken to dryness on a rotary evaporator, dissolved in 1.0 ml of starting buffer and analysed on a Spinco Model 220 amino acid analyzer according to the method of Spackman et al (1958).

(c) High Voltage Electrophoresis

High voltage electrophoreses were carried out for 45 minutes on a Gilson High Voltage Electrophorator in pyridine-acetate buffer (10%; 0.4%) pH 6.5 on Whatman #3 filter paper. Peptide material was located by dipping the paper in a ninhydrin solution prepared as follows:
(i) 1% ninhydrin (Pierce Chemicals) in acetone
(ii) 5 g cadmium acetate, and 250 ml acetic acid dissolved in 500 ml distilled water
(iii) 85 ml of (i) and 15 ml of (ii) were mixed just prior to use and the electrophoretogram was developed at room temperature in a fume hood.

IV. Chemical Modifications of Ferredoxin

(a) Iron Sulfide Free Ferredoxin (TCA-Fd)

Iron sulfide free ferredoxin was prepared according to the method of Tanaka et al (1964). Thirty mg of ferredoxin were dissolved in 4.0 ml of distilled water and 2.0 ml of a 15% solution of trichloroacetic acid and 0.1 ml of 2-mercapto-ethanol were added. The mixture was allowed to stand 1 hr at 4 C. The mixture was sedimented in a clinical centrifuge and the precipitate (TCA-Fd) was washed consecutively with 5.0 ml of distilled water, 5.0 ml of 95% ethanol and 5.0 ml of ether. The final precipitate was dried under vacuum.

(b) Performic Acid Oxidized Ferredoxin (OFd)

The cysteine residues of iron sulfide free ferredoxin were converted to cysteic acid by oxidation with performic acid. Fifteen mg of TCA-Fd were dissolved in 1.0 ml of concentrated
formic acid. To this solution was added 2.0 ml of performic acid (prepared by mixing 9.0 ml of formic acid with 1.0 ml of 30% hydrogen peroxide). The mixture was allowed to react for 4 hr at -10°C and was then lyophilized.

(c) Carboxamidomethylated Ferredoxin (CAM-Fd)

TCA-Fd was dissolved to a concentration of 1-2 mg/ml in distilled water adjusted to pH 8.0 with dilute NaOH. Reduction of cysteiny1 residues was ensured by adding an 80-fold molar excess of 2-mercaptoethanol followed by incubation at 47°C for 4 hr. The protein was precipitated by gradually acidifying the solution with 4 N HCl. The precipitate was centrifuged, re-suspended in water, and an equal amount (w/w) of iodoacetamide was added. The pH was then adjusted to 8.0 with dilute NaOH and alkylation was allowed to occur for 10 min at room temperature. The pH was again lowered with 4 N HCl and the precipitated protein was collected by centrifugation and washed once with distilled water before being dissolved in the appropriate buffer.

V. Immunization Procedure.

Purified C. pasteurianum ferredoxin used for production of antisera was purchased from Worthington Biochemical Co. Antisera were raised in rabbits to native ferredoxin and its performic
acid oxidized derivative. Five mg of antigen were dissolved in 0.5 ml physiological saline and emulsified in an equal volume of complete Freund's adjuvant (Difco). Rabbits were anaesthetized with an intravenous injection of 1.0 ml nembutal (Hyland Co.) and 0.2 ml of the adjuvant preparation was injected into a foot pad of each extremity and 0.2 ml was injected intramuscularly in the left hind leg. The following day, 1.0 mg of alum precipitated antigen, prepared according to the method of Kabat and Mayer (1961), was injected intravenously in an ear vein. After 4 weeks, ear bleeds were performed weekly and the serum was stored in 50 ml batches after heating at 55 C for 30 min to destroy complement activity. Titers were found to be stable over a six-month period, at which time a booster injection of 1 mg of antigen in 0.5 ml complete Freund's adjuvant was administered in the left hind leg. Merthiolate at a concentration of 1:10,000 was added to the sera as a preservative.

VI. Complement Fixation

The complement fixation reaction was used for initial studies on the antigenic properties of ferredoxin and its cysteine-modified derivatives. Freeze-dried guinea pig serum (3.0 ml quantities) purchased from Hyland Co. was used as the source of complement. The serum was reconstituted for each test in the diluent provided and further dilutions were made in veronal buffered saline, between 1:25 and 1:250, between 1:50 and 1:500 or between 1:75 and 1:750, depending
on the batch of guinea pig serum used. The veronal buffered saline used for all dilutions of antigen, antiserum, complement and hapten was prepared according to the method of Brooksby (1952).

The complement fixation titers of the two antisera were determined by using serial dilutions of complement between the values given above, doubling dilutions of antiserum between 1:10 and 1:320 and one dilution of antigen (0.5 μg). The antigen, antiserum and complement were all used in 1.0 ml quantities per tube. A similar test to check the anticomplementary activity of the antiserum was set up simultaneously using 1.0 ml of veronal buffered saline instead of antigen. The tubes were mixed by shaking and incubated overnight at 4°C. A 2% suspension of twice-washed sheep erythrocytes in veronal buffered saline was sensitized by mixing them with an equal volume of 1:50 dilution of a standard solution of sheep hemolysin (Difco), for 10 min. This preparation was added in 1.0 ml amounts to the test series which were then incubated at 37°C for 30 min in a water bath. Tubes were shaken once at 15 min. Following the 30 min incubation, unhemolyzed red cells and cell debris were sedimented by centrifugation at 1800 x g for 10 min, and the supernatants were read on a Klett colorimeter with a green filter. End points were calculated as 50% hemolysis on a probit plot of actual percentages of the Klett readings according to the method described by Wright (1963). The titer of the native ferredoxin antiserum was calculated to be 1:40 and that of the oxidized ferredoxin antiserum 1:200. No anticomplementary activity could be detected at these dilutions of antiserum.
The degree of cross-reaction between the two antisera and the four antigen preparations (native Fd, TCA-Fd, OFd and CAM-Fd) was determined using the complement fixation reaction. In this case, a constant dilution of antiserum was used throughout (1:40 for native Fd antiserum and 1:200 for oxidized ferredoxin antiserum, as previously determined), mixed with doubling dilutions of each antigen ranging from 12 μg to 0.25 μg at each of the ten dilutions of complement, each in a total volume of 1.0 ml. The incubation times, temperatures and assay method were identical with those used for determining the titers of the antisera. Endpoints were calculated as the 50% hemolysis point from probit plots as described above.

VII. Quantitative Precipitin Reaction

The degree of cross-reaction was also determined using the quantitative precipitin reaction. The antiserum to OFd at a dilution of 1:4 was dispensed in 0.5 ml quantities to a series of tubes. The various antigen preparations, also in a volume of 0.5 ml, were added in amounts varying from 0.25 to 25 μg. Each test was performed in triplicate. A negative control was set up using 0.5 ml of saline instead of antigen. The diluent used throughout was 0.9% saline. Tubes were mixed and incubated for 1 hr in a 37 °C water bath and then overnight at 4 °C. The following day, the precipitated were sedimented in a clinical centrifuge and washed twice with 0.9% saline.
The washed precipitates were dissolved in 0.1 N NaOH and absorbances at 280 μ, 1.0 cm light path, were read on a Beckman DBG spectrophotometer.

VIII. Peptide Synthesis

The COOH-terminal octapeptide of C. pasteurianum ferredoxin was synthesized using the solid phase method of Merrifield (1964) as described by Stewart and Young (1969). The chloromethylated polystyrene-divinylbenzene copolymer resin was purchased from Biorad Co., and the protected t-butyloxy carbonyl (t-boc) amino acids were purchased from Mann Research Biochemicals and Sigma Chemical Co. One gram of resin was used for the synthesis procedure, and protected amino acids were added in a 3.5 fold molar excess of the amount of the initial amino acid attached, except for asparagine, which was added in a 5-fold molar excess. The peptide was cleaved from the resin by bubbling anhydrous HBr (Matheson Co.) through a suspension of the resin in 10.0 ml of anhydrous trifluoroacetic acid for 90 min; this procedure also removed the protecting O-benzyl group from the glutamic acid residue. Trifluoroacetic acid was removed by flash evaporation and lyophilization. The peptide was purified further by column chromatography on Dowex Ag-1-X2 resin (200-400 mesh-Biorad Co.), according to the method of Konigsberg and Hill (1962). The prepared resin was poured into a 1.5 cm diameter column to a height of 27 cm and equilibrated
with N-ethyl morpholine-acetate buffer (0.2 M in acetate) at pH 8.6. The peptide was applied to the column in a volume of 2.0 ml of this buffer. The column was developed with a gradient; the mixing chamber contained 50 ml of the pH 8.6 buffer and the reservoir contained 50 ml of 1.0 M pyridine-acetate buffer at pH 6.56. After the gradient was completed, 50 ml of 2 M pyridine-acetate buffer at pH 4.05 was run through the column. A flow rate of 20 ml/hr was maintained and 4.0 ml samples were collected on an LKB fraction collector.

A 0.2 ml aliquot was taken from every other fraction for determination of peptide content by the quantitative ninhydrin method of Hirs et al. (1966) after alkaline hydrolysis was carried out according to the method of Stewart and Young (1969). The peptide-containing fractions were pooled and lyophilized. The dried peptide samples were dissolved in 2.0 ml of distilled water. The peptides were checked for purity by high voltage electrophoresis and their compositions determined using the amino acid analyzer.

IX. Hapten Inhibition Studies

The haptenic activity of the synthesized peptide was tested by inhibition of complement fixation and by inhibition of precipitation. Both tests were carried out basically as described by Gerwing and Thompson (1969). For inhibition of complement fixation, native Fd and OFd antisera at 1:40 and 1:200 dilutions respectively were mixed in
1.0 ml volumes with amounts of peptide varying between 0.25 and 25 μg in 0.10 ml quantities, each over a range of 10 complement dilutions. A positive control was set up using 0.1 ml of veronal buffered saline instead of peptide for one series of tubes. The tubes were incubated for 1 hr in a 37 C water bath and then overnight at 4 C. The following day, 1.0 ml of the homologous antigen (0.5 μg) and 1.0 ml of the appropriate dilution of complement was added to each tube. Incubation was carried out overnight at 4 C. On the third day the hemolysin-erythrocyte indicating system was added and hemolysis determined as previously described. The amount of inhibition was calculated as the difference in percentage hemolysis between the tubes in the test series and those of the positive control containing the same dilution of complement when compared with a no antigen control, which was taken at 100% hemolysis. The tubes in the positive control on either side of the 50% hemolysis point were chosen for calculation.

To determine the degree of inhibition of precipitation caused by the peptide, a modification of the procedure described by Gerwing and Thompson (1969) was used. Varying amounts of hapten between 500 and 5 μg, in a volume of 0.1 ml were mixed in triplicate with a constant amount of antiserum in 0.5 ml at a 1:4 dilution. Tubes were incubated for 1 hr in a 37 C water bath and then antigen at a concentration previously determined to be slightly in excess of optimum was added in 0.5 ml quantities. Incubation was continued for another hour at 37 C. The precipitates formed were sedimented, washed once with saline and
dissolved in 1.0 ml of 0.1 N NaOH and the absorbances read at 280 μμ μμ, 1.0 cm light path, on a Beckman DBG spectrophotometer.

X. Equilibrium Dialysis

(a) Purification of Antibody.

Specific antibodies directed against native ferredoxin and its performic acid oxidized derivative were purified from their respective antisera using a specific immunoadsorbent. An OFd immunoadsorbent was used to obtain antibodies from each serum. The method of preparation of the immunoadsorbent and purification of antibody was basically that of Robbins et al (1967). Seventy-five mg of OFd were covalently bonded to 1.0 g of bromoacetyl cellulose (prepared by reaction of bromoacetic acid and bromoacetyl bromide with 1.0 g of Whatman powdered cellulose) in 0.15 M citrate-phosphate buffer pH 4.6. Before adsorption, 75 ml of each serum was clarified by centrifugation at 20,000 x g for 1 hr, and sedimented debris and floating lipid material were removed. The cellulose conjugate was then dispersed in the serum, and the suspension was stirred at 4 °C for 2 hr. Then the adsorbed conjugate was removed by centrifugation at 20,000 x g for 20 min, and all supernatant serum was drained off. The conjugate was washed with 0.15 M NaCl and recentrifuged until the absorbance of the supernate at 280 μμ μμ μμ was less than 0.08. The antibody was eluted by stirring
the complex in 10-15 ml of 0.1 M acetic acid (pH 2.8) for 1 hr at 37 C. The adsorbent was removed by centrifugation at 20,000 \( x \) g for 20 min, and the supernatant fluid containing the antibody was dialyzed against 350-700 volumes of 0.1 M NaCl-0.01 M Tris-HCl buffer pH 7.0. The purified antibody was quantitated by the Lowry method as described by Kabat and Mayer (1961), and the titer of the solution was determined by complement fixation. Purified antibody was stored at 4 C in the Tris buffer described above to which 60 \( \mu \)g merthiolate ml had been added. After washing once with 0.1 M acetic acid, the immunoadsorbent was stored at 4 C suspended in 0.15 M phosphate buffer pH 7.6.

(b) Purification of Non-specific Gamma Globulin

Gamma globulin, for use as a control, was purified from non-specific rabbit serum by precipitation with 50% ammonium sulphate, dialysis, and subsequent column chromatography on DEAE cellulose with 0.05 M phosphate buffer pH 7.5 according to the method of Fujio et al (1968a). The gamma globulin was stored as a lyophilized powder. For use in equilibrium dialysis, small amounts of the powder were dissolved in PBS (phosphate buffered saline: 0.02 M phosphate buffer pH 7.0, 0.15% NaCl and 0.02% sodium azide) and exhaustively dialyzed at 4 C against the same buffer. The protein concentration was determined by the Lowry method.
(c) (1-^{14}C)-Acetylation of the Peptide

The preparation of the (1-^{14}C)-acetylated peptide was carried out according to the method of Fujio et al. (1968b). The (1-^{14}C)-acetic anhydride (5mCi/mM) was purchased from New England Nuclear Corp. in 0.10 Ci breakseal tubes. The tube was frozen in an acetone-dry ice bath and 1.5 ml of anhydrous benzene was placed over the vacuum seal. Once the seal was broken, the benzene was drawn into the tube, dissolving the labelled anhydride. The benzene solution was warmed slightly and drawn out of the tube with a syringe and transferred to a small screw-capped vial. Fourteen umoles of the peptide were dissolved in 1.5 ml of 1.0 M sodium acetate adjusted to pH 8.0 with NaOH. A 20-fold molar excess of labelled acetic anhydride in benzene was layered on top of the aqueous peptide solution in a small screw-capped vial. After 6 hr at 4 C, the aqueous phase was withdrawn and passed through a 2.5 cm x 40 cm column of Sephadex G-15 (Pharmacia) equilibrated with 50% acetic acid. The column was eluted with the same solvent at 25 C. Fractions of 4.5 ml were collected and 0.10 ml from every second tube were mixed with 10.0 ml of scintillation fluid and the radioactivity measured on a Nuclear Chicago Scintillation Counter Model 750. The scintillation fluid was prepared as follows: 42.0 ml Liquifluor (New England Nuclear Corp.) and 383.2 ml methanol dissolved in 547.8 ml toluene.

The peptide-containing fractions were pooled and taken to
Dryness by flash evaporation, using a special dry-ice acetone trap. The peptide was redissolved in 2.0 ml distilled water, lyophilized and then dissolved in PBS. Duplicate 0.01 ml samples were mixed with 10.0 ml of the scintillation fluid and the radioactivity was measured to determine the amount of label present on the peptide. A small amount of peptide was also taken for amino acid analysis so that specific activity could be established.

(d) Dialysis Procedure

Equilibrium dialysis experiments were set up using pencil dialysis tubing (Visking-Union Carbide-1 Cm) and small screw-capped vials. Phosphate-buffered saline was used as diluent throughout. Purified antisera to both native ferredoxin and oxidized ferredoxin, containing between 300 to 400 µg protein/ml, were added to individual dialysis tubes in 1.0 ml quantities. Controls were set up using 1.0 ml volumes of PBS and non-specific gamma globulin, also in individual dialysis tubes. The dialysis sacs were immersed individually in 10.0 ml of the labelled peptide solution, at concentrations of 0.002 and 0.001 µmoles/ml. The vials were placed in a shaker at 4°C, and dialysis was allowed to continue for 4 days, at which time equilibrium was reached, as established by measurement of the PBS control. Then 0.10 ml samples from inside and outside each dialysis membrane were taken and the radioactivity was measured. Duplicate counts per minute were estimated from 10 min counts of each sample.
RESULTS AND DISCUSSION

I. Preparation of Ferredoxin

The antigenicity of ferredoxin from *Clostridium pasteurianum* was studied in an attempt to determine the antigenic binding sites of the protein. This molecule was considered a good subject for such a study since the amino acid sequences of ferredoxins from several related species which have been published (Tsunoda and Yasunobu, 1968), show extensive sequence homology, and because it is a small protein which should possess relatively few antigenic determinants. For use in immunological testing of the antisera, ferredoxin was purified from cell preparations of *C. pasteurianum* grown in the laboratory. Four 30 litre cultures were grown in a completely synthetic medium and harvested by continuous flow centrifugation. The yield of cell paste for the four batches of culture is given in Table 1. Using the continuous flow method of centrifugation as described in the Methods, a complete culture could be harvested in four hours without stopping the centrifuge. The decrease in optical density at 650 μ between the culture and the effluent was calculated to be 99.9%. Ferredoxin was purified from each batch of cell separately. The purification procedure takes advantage of the fact that ferredoxin is soluble in a 50% aqueous solution of acetone while 99% of the remainder of the cellular constituents are not; and of the fact
<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Wt. Cell Paste</th>
<th>Wt. Ferredoxin</th>
<th>390 μM/280 μM ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>301 g.</td>
<td>56 mg.</td>
<td>0.78</td>
</tr>
<tr>
<td>2</td>
<td>432 g.</td>
<td>62 mg.</td>
<td>0.73</td>
</tr>
<tr>
<td>3</td>
<td>205 g.</td>
<td>35 mg.</td>
<td>0.74</td>
</tr>
<tr>
<td>4</td>
<td>350 g.</td>
<td>42 mg.</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 1. Yield of cell paste and purified ferredoxin with the 390 μM/280 μM ratios found for the four batches purified.
that ferredoxin is extremely electronegative (pl = 3.7, Lovenberg, et al., 1963) and will adsorb to an anion exchange resin under conditions which allow most of the remaining contaminating material to be washed out. Final purification was achieved by ammonium sulphate fractionation. The final product was dark brown and readily soluble in distilled water or buffer. The yield of purified ferredoxin from each of the four batch cultures is also shown in Table 1.

Three criteria were used to establish the degree of purity of the final preparations:

(i) The optical absorption spectrum was determined for each sample. Ferredoxin should show absorption maxima at 390 m\(\mu\) and 280 m\(\mu\), and the ratio between the two is an indication of the relative purity of the preparation. Figure 4 shows a representative absorption spectrum of a purified preparation which exhibits the typical maxima. The 390 m\(\mu\)/280 m\(\mu\) ratios of the four preparations are also listed in Table 1. They vary from 0.72 to 0.78, which are in fair agreement with the reported value of Buchanan et al. (1963) of 0.80 and with that of the commercial preparation obtained from Worthington Biochemical Corp., with a ratio of 0.75.

(ii) Only a single, negatively charged ninhydrin-positive spot could be detected in each sample after high voltage electrophoresis.

(iii) The amino acid composition of each sample was determined. The composition of a representative sample is listed in Table 2, and, for comparison, the composition reported by Mortenson (1964) is also given. The fact that leucine, histidine and arginine
Fig. 4. Spectrum analysis of *C. pasteurianum* ferredoxin.
### GM/100 GM Protein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fd*</th>
<th>Fd</th>
<th>TCA-Fd</th>
<th>O-Fd</th>
<th>CAE-Fd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>3.0</td>
<td>2.8</td>
<td>2.8</td>
<td>2.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>17.0</td>
<td>15.3</td>
<td>16.8</td>
<td>17.1</td>
<td>16.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.3</td>
<td>1.9</td>
<td>2.45</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Serine</td>
<td>4.9</td>
<td>5.1</td>
<td>5.8</td>
<td>2.7</td>
<td>7.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.6</td>
<td>7.6</td>
<td>11.2</td>
<td>9.0</td>
<td>8.2</td>
</tr>
<tr>
<td>Proline</td>
<td>6.3</td>
<td>6.5</td>
<td>6.3</td>
<td>7.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Glycine</td>
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<td>9.5</td>
<td>10.3</td>
<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
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<td>13.4</td>
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</tr>
<tr>
<td>(\frac{1}{2}) Cysteine</td>
<td>5.3</td>
<td>8.7</td>
<td>6.8</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>Cysteic acid</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Carboxamido-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.5</td>
</tr>
<tr>
<td>methylcysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
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<td>12.0</td>
<td>11.7</td>
<td>12.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>9.8</td>
<td>8.7</td>
<td>9.4</td>
<td>8.3</td>
<td>9.4</td>
</tr>
<tr>
<td>Tyrosine</td>
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<td>1.6</td>
<td>0.84</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.6</td>
<td>2.7</td>
<td>1.9</td>
<td>2.2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 2. The amino acid compositions of the various antigenic preparations used in these studies.

* Amino acid composition for native Fd reported by Mortenson (1964).
could not be detected in amounts exceeding 0.1 g per 100 g protein indicated that the preparations were virtually free of peptide contaminants.

II. Antisera

Carpenter (1965) reports that the average molecular weight for antigenic substances is 10,000; with the smallest antigens found to date being a synthetic homopolymer of glutamic acid with a molecular weight of 4100 (Sela, 1966), and a phenylisocyanate of clupein of molecular weight 5000 (Carpenter, 1965). Ferredoxin, with a molecular weight of 5500 approaches this limit of size for antigenicity. Therefore, the first problem presented was to determine if ferredoxin could evoke an antibody response in rabbits. Both native ferredoxin and its performic acid oxidized derivative were used as antigens to test what effect the modification of cysteine residues had on the production of antibody. Individual rabbits were immunized with adjuvant preparations of each antigen. A commercial preparation of ferredoxin was used for the production of antisera and oxidized ferredoxin was prepared from this as described in Methods. Serum was collected and the presence of specific antibodies was tested for by means of the complement fixation reaction. This method was chosen for initial determination of antibody since it is the most sensitive in vitro method of detection available, and will indicate an antigen-
antibody reaction when other methods, such as hemagglutination or precipitation are negative (Kabat and Mayer, 1962).

Each antiserum was assayed with one concentration of its homologous antigen over a range of 10 complement dilutions, which were previously found to give a good range of hemolysis with the indicating system. Endpoints were calculated as the 50% hemolysis level, since the reaction has a linear relationship with complement concentration in this range, while toward 100% hemolysis the relationship begins to level off. Both native Fd and OFd were found to be antigenic in rabbits. Oxidized ferredoxin proved to be a stronger antigen, producing a titer of 1/200, while native Fd antiserum had a titer of 1/40.

The precipitating abilities of the two antisera were also tested. In this case, antiserum at a 1/4 dilution was mixed with varying concentrations of homologous antigen and the resulting precipitates were removed, washed and dissolved in dilute NaOH. Quantitation involved determining the absorbance of the solution at 280 nm. Native Fd antiserum even when undiluted, failed to precipitate its homologous antigen, possibly due to its low titer. OFd antiserum did precipitate its homologous antigen, and the results are presented graphically in Figure 5.

III. Modification of the Cysteine Residues of Ferredoxin

After establishing the antigenicity of ferredoxin, the areas of
Fig. 5. Quantitative precipitation reactions of antiserum against OFd at 1:4 with various antigenic preparations: 0-0 native ferredoxin; Δ - Δ TCA-Fd; 0-0 OFd.
the molecule responsible for binding specific antibody were investigated. The general method used for determining antigenic determinants, involving isolation of haptenic peptides from a proteolytic digest, was not found to be practicable for the ferredoxin molecule. Although the protein contains one residue which should be susceptible to trypsin (Lys\textsuperscript{3}) and two which should be susceptible to chymotrypsin (Tyr\textsuperscript{2} and Phe\textsuperscript{30}), (Figure 6), the native molecule is completely resistant to hydrolysis by these enzymes (Tanaka, 1963). The performic acid oxidized protein can be digested with chymotrypsin, if digestion is prolonged and a large amount of enzyme is used (35\% by weight). In this case three peptides are produced, an NH\textsubscript{2}-terminal dipeptide, and two other peptides of almost equal size (28 and 25 residues) and charge. The first peptide is too small to be immunologically useful and the latter two are too similar in size and charge to make separation feasible. The cysteine residues in the ligand-free apoprotein can be modified to make them susceptible to trypsin or chymotrypsin, and this method in fact, was used to isolate peptides for sequence analysis (Tanaka et al., 1966), but was not considered useful until the role of cysteine in antigenicity was determined.

Therefore, the second common method used for determining antigenic binding sites was chosen for initial studies. This involves modification of specific residues in the protein and subsequent testing of the modified antigen for its ability to bind antibody. Ferredoxin is unique in that it contains 8 cysteine residues, a high proportion
Figure 6. Amino Acid Sequence of *C. pasteurianum* Ferredoxin.

Suggested antigenic sequences are underlined.

Upper Case: C-terminal octapeptide.
for such a small molecule. These are not involved in disulfide bonds
and are spaced relatively evenly in two regions of the molecule
(Figure 6). Three derivatives of native ferredoxin were prepared
in order to determine the participation of cysteine residues in the
antigenicity of the protein. In the native molecule, the 8
molecules of iron and hydrogen sulfide complexed to each protein
(Buchanan et al., 1963) are postulated to be complexed to the protein
through the sulfhydryl groups of the cysteine residues (Ehrenberg
et al., 1967) so that removal of the iron and hydrogen sulfide would
be expected to disrupt the environment of the cysteine residues of the
native molecule. Treatment of native ferredoxin with 5% trichloro-
acetic acid caused immediate bleaching and precipitation of the protein
accompanied by evolution of \( \text{H}_2\text{S} \), phenomena associated with removal
of the inorganic ligands from the protein (Tanaka et al., 1963). From
30 mg of native ferredoxin, 23 mg of white apoferredoxin (TCA-Fd)
were obtained which were readily soluble in distilled water or buffer.
The only precaution taken to ensure that disulfide bond formation did
not occur in the TCA-precipitated product, was the addition of 0.10
ml of 2-mercaptoethanol to the original precipitation mixture. This
precaution seemed adequate, since insoluble aggregates were never
observed in the preparation, and its immunological reactivity remained
uniform from test to test. Any alterations occurring due to di-
sulfide formation would be expected to decrease its immunological
reactivity and, as will be shown below, this did not occur.
After hydrolysis with 6 N HCl, the amino acid composition of a sample of TCA-Fd was determined on a Beckman Amino Acid Analyzer. The composition is listed in Table 2. After hydrolysis, amino acid analysis showed that the cysteine residues were totally converted to cysteic acid. Since, as will be reported below, this sample of TCA-Fd reacted with iodoacetamide, indicating the presence of free sulfhydryl groups, and since its immunological behaviour differed quantitatively from an authentic sample of oxidized ferredoxin, it was assumed that the oxidation of the sulfhydryl groups was the result of hydrolysis, possibly due to the presence of trace amounts of trichloroacetic acid.

Two further derivatives were prepared from the TCA-treated product; one in which the cysteine residues were converted to cysteic acid by performic acid oxidation to place a strong negative charge on the residues; and one in which the sulfhydryl groups were alkylated with iodoacetamide to place a bulky neutral group on the residues. Both preparations were readily soluble in distilled water and buffer. The degree of modification of the cysteine residues was determined by amino acid analysis and calculation of the amounts of cysteic acid and carboxamidomethylcysteine, respectively, which were recovered. The amino acid compositions of the two derivatives are listed in Table 2, and show that the reaction in each case was virtually complete, since no free cysteine could be recovered.
IV. Immunological Reactivity of Ferredoxin and its Derivatives

The extent of cross-reaction between the two antisera and the four different ferredoxin preparations (native Fd, TCA-Fd, OFd and CAM-Fd) was determined by complement fixation. Native ferredoxin antiserum at a dilution of 1/40 was mixed with varying amounts of each preparation over a range of 10 complement dilutions. Each test was performed at least twice and they were found to agree within 5%. The endpoints were calculated as the 50% hemolysis point from probit plots of actual percentages. As shown in Figure 6, cross-reaction occurred in all cases. The results are surprising however, in that the maximal reactions were obtained with the iron sulfide-free and alkylated derivatives, rather than with the supposedly homologous antigen, native ferredoxin. The fact that the two neutral, ligand-free preparations appear to react maximally with the antibody may indicate that in vivo the native molecule is modified before antibodies are produced against it.

OFd antiserum was assayed at a 1/200 dilution in a similar manner to the native ferredoxin antiserum, and again, as shown in Figure 7, cross-reaction occurred in all cases, with the homologous system giving the maximal reaction. The good cross-reaction observed with OFd antiserum is interesting in light of the results of Brown (1962) studying the antigenicity of performic acid oxidized ribonuclease. The cysteic acid residues would be expected to be highly immuno-
Fig. 7. Complement fixation reaction of antiserum against native ferredoxin at 1:40 with various antigenic preparations; ø - ø native Fd; Δ - Δ TCA-Fd, Δ - Δ alkylated Fd; 0 - 0 OFd.
Fig. 8. Complement fixation reactions of antiserum against O-Fd at 1:200 with various antigenic preparations: 
- native Fd, 
- TCA-Fd, 
- alkylated Fd, 
- OFd.
genic since they would be novel to the antibody synthesizing cells, but he found that cysteic acid was not highly immunogenic, with only one of eight cysteic acid residues and none of the two methionine sulfoxide residues appearing in the sequences of the two haptenic peptides he isolated.

With this antiserum, the cross-reaction data were confirmed by the quantitative precipitation reaction using native Fd and TCA-Fd as heterologous antigens, (due to lack of material CAM-Fd was not tested). Again, cross-reaction was obtained in all cases, with the homologous system demonstrating the maximal reaction, as shown in Figure 5, while native Fd and TCA-Fd gave lesser but roughly equal reactions, precipitating 50% as much antibody as OFd.

The cross-reaction data from both complement fixation and precipitation tests indicate that at least two antigenic sites exist on the ferredoxin molecule which do not directly involve cysteine, since two binding sites on the antigen are necessary for lattice formation and precipitation. The possible involvement of cysteine in antigenic sites cannot be completely excluded, however, since differences in reactivity do exist between the four preparations and the two antisera; although these differences may be due at least in part, to unavoidable configurational changes concomittant with chemical modification. The cross-reaction that is observed is assumed to be the result of reaction of the respective derivatives and not due to participation of unmodified material, since amino acid analyses of
each preparation indicated virtually complete conversion to the respective modified derivative.

V. Solid Phase Synthesis of the COOH-Terminal Octapeptide

The results from cross-reaction between OFd and native Fd antisera with the four preparations of ferredoxin indicate that at least two antigenic sites are present on the protein which do not have cysteine residues in their sequences. Assuming a minimum antigenic size of 5 amino acid residues, the areas of the protein which could act as antibody binding sites are somewhat limited. The areas encompassing residues Cys\(^7\) to Cys\(^{19}\) and Cys\(^{37}\) to Cys\(^{48}\) can be eliminated, leaving three regions, from Ala\(^1\) to Ser\(^7\), Pro\(^{18}\) to Thr\(^{36}\) and Pro\(^{49}\) to Glu\(^{55}\), which possibly possess antigenic activity (Figure 8). At this point, it was considered advantageous to obtain peptides by solid phase synthesis rather than by enzyme digestion of a cysteine-modified derivative of ferredoxin, since the synthetic method provides a purer product in less time with greater yield. The first area chosen for study was the COOH-terminal octapeptide of sequence pro-val-gly-ala-pro-val-glu-glu because it is relatively small though large enough to possess antigenic activity, it is quite hydrophobic, it is easily synthesized (containing only one residue with a reactive side group), and finally, the COOH-terminal portions of sperm whale myoglobin and hen egg-white lysozyme have been implicated in antibody binding.
Solid phase synthesis of peptides is a recent technique described by Merrifield (1964). It involves the stepwise addition of amino acids to a growing peptide chain which is attached at its COOH-terminal end to an insoluble support, in this case a resin bead of chloromethylated polystyrene-divinylbenzene copolymer. The first amino acid, as its triethylammonium salt, is attached to the resin by an ester link via the chloromethyl groups. The NH$_2$-terminal end of each amino acid is blocked by a tert-butyloxycarbonyl group (t-boc) to eliminate side reactions. Attachment of the growing peptide to an insoluble support facilitates removal of solvents and excess reagents by simple filtration, eliminating wasteful and time-consuming purification procedures at each step. The t-boc group of the terminal amino acid is removed by 1 N HCl in glacial acetic acid and the next amino acid is coupled to the freed NH$_2$-terminal group using dicyclohexylcarbodiimide (DCC) as a dehydrating agent in forming the peptide bond. The amino acids asparagine and glutamine are coupled as their p-nitrophenyl esters without the mediation of DCC, in which case the reaction time was increased from 2 to 5 hours and a 5-fold instead of a 3.5-fold molar excess of the amino acid was added. The peptide is built up stepwise in this manner, with reactive $R'$ groups suitably blocked, generally by a benzyl group. After the last amino acid is attached, the peptide is cleaved from the resin by anhydrous HBr in anhydrous trifluoracetic acid. This treatment also removes the final t-boc group and the O-benzyl protective
Fig. 9. Flow chart of Solid Phase Peptide Synthesis.

\[
\text{CH}_3\text{OH} \quad R_1 \quad \text{O} \\
\text{CH}_3\text{C} \text{-O-C-N-C-C-O-} + \text{Cl-CH}_2\text{-POLYMER} \\
\text{Boc amino acid} \quad \Downarrow \quad \text{chloromethyl polymer} \\
\text{CH}_3\text{C} \text{-O-N-C-C-O-CH}_2\text{-POLYMER} \\
\text{Boc amino acyl polymer} \\
\Downarrow \quad \text{HCl—dioxane; Et}_3\text{N} \quad \text{DEPROTECT; NEUTRALIZE} \\
\text{CH}_2 + \text{CO}_2 + \text{HN-C-C-O-CH}_2\text{-POLYMER} \\
\text{isobutylene} \quad \text{amino acyl polymer} \\
\Downarrow \quad \text{Boc amino acid} \quad \text{diimide} \quad \text{COUPLE} \\
\text{CH}_3\text{C} \text{-O-C-N-C-C-O-C-N-C-C-O-CH}_2\text{-POLYMER} \\
\text{Boc peptide polymer} \\
\Downarrow \quad \text{HBr—F}_2\text{CCOOH} \quad \text{CLEAVE.} \\
\text{CH}_2 + \text{CO}_2 + \text{HN-C-C-O-H} + \text{Br-CH}_2\text{-POLYMER} \\
\text{isobutylene} \quad \text{peptide} \\
\]

*Reproduced from Solid Phase Peptide Synthesis by John Stewart and Janis Young, p. 3.*
group present on glutamic acid of the peptide sequenced in this instance. The sequence of steps is represented diagrammatically in Figure 10. The amount of the first amino acid attached to the resin is determined by hydrolysis of a weighed sample followed by amino acid analysis, and from this the amount of each subsequent amino acid and DCC to be added is calculated.

The cleaved peptide was purified by ion-exchange chromatography and the peak containing the peptide with the best amino acid ratio was identified by amino acid analysis. The elution profile is shown in Figure 11, and the amino acid composition of the purified peptide is listed in Table 3. The composition is very close to the theoretical. This, and the fact that only one ninhydrin-positive spot could be detected by high voltage electrophoresis indicated that the peptide was essentially homogeneous. Of a possible 0.72 micromoles, 0.267 micromoles of glutamic acid was attached to the resin (36.8% of the theoretical), and 100 micromoles of peptide was recovered, which is 37.5% of the theoretical yield calculated from the amount of attachment.

VI. Immunological Testing of the Synthetic Peptide

The haptenic activity of the synthetic peptide was determined by testing its ability to inhibit complement fixation. Varying amounts of the peptide were added to a series of tubes containing native Fd antiserum and OFd antiserum at 1/40 and 1/200 dilutions respectively.
Fig. 10. Elution profile of the COOH-terminal peptide. The arrow indicates time of the buffer change after completion of the gradient. The peak eluting at about 105 ml. contained the octapeptide.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>umoles</th>
<th>molar ratio</th>
<th>residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>1.121</td>
<td>2.19</td>
<td>2</td>
</tr>
<tr>
<td>Proline</td>
<td>1.033</td>
<td>2.02</td>
<td>2</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.476</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.547</td>
<td>1.07</td>
<td>1</td>
</tr>
<tr>
<td>Valine</td>
<td>1.025</td>
<td>2.00</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3. Amino acid analysis of the C-terminal octapeptide of ferredoxin, sequence: pro-Val-gly-ala-pro-Val-gln-glu.
After incubation, homologous antigen and complement were added, and then the degree of fixation was determined from the amount of hemolysis. The degree of inhibition was calculated as the difference in percentage hemolysis between the test series and the tube containing the same concentration of complement in the positive control (not containing hapten) when compared to a no hapten-no antigen control. The averaged results of these tests are shown graphically in Figure 11. The octapeptide exhibited inhibitory activity with both sera, to the extent of 25% with native Fd antiserum and 36% with OFd antiserum. In all cases, the hapten proved more inhibitory at 25 μg than at 50 μg; this phenomenon was consistent from one set of complement fixation tests to the next, and was specific for the ferredoxin system, since no increased fixation could be detected in a non-homologous system consisting of lysozyme and anti-lysozyme serum when 50 μg of hapten was used.

The ability of the hapten to inhibit the precipitation reaction between OFd and its homologous antibody was also tested. In initial studies, hapten in concentrations ranging from 2.5 to 50 μg in 0.1 ml were added to triplicate samples of antiserum and incubated for 1 hr, then homologous antigen was added and the incubation continued overnight. By this method, a maximum inhibition of only 7% was obtained. Therefore, the test was modified: hapten was incubated with antiserum for 1 hr, with a range from 500 to 2.5 μg, and then antigen was added and incubation was continued. After 1 hr, the precipitates
Fig. 11. Inhibition of complement fixation by the COOH-terminal octa-peptide when tested against native ferredoxin and OFd and their homologous antisera. 0-0 and 0-0 represent dilutions of 1:100 and 1:125 of complement with OFd and anti-OFd. Δ - Δ and Δ - Δ represent the same dilutions of complement with native ferredoxin and its homologous antigen.
were centrifuged, washed, dissolved in dilute NaOH and the absorbances of the solutions read at 280 μm. The results of the averaged triplicate samples are shown in Figure 13. The degree of inhibition was still quite low and the maximum level was not reached, but the results were reproducible to within 0.5%.

Finally, the reactivity of the peptide was tested by direct binding studies employing the equilibrium dialysis technique. With this method, antiserum is separated from the hapten by a semipermeable membrane which allows passage of the small hapten but not of the antibody. The antibody solution, in a dialysis sac, is immersed in a solution of hapten, and the hapten is allowed to reach equilibrium on each side of the membrane. After equilibrium is reached the concentration of hapten on each side of the membrane is measured. The difference between the amount of hapten found on the antibody side of the membrane and the concentration on the outside of the dialysis sac is a measure of the amount of hapten bound to antibody.

In order to measure hapten concentration, a radioactive label, 1-¹⁴C-Acetyl-, was attached to the NH₂-terminus of the octapeptide. This was accomplished by layering a 20-fold molar excess of labelled acetic anhydride in anhydrous benzene over an aqueous solution of the peptide. When the reaction was completed, the excess label was separated from the octapeptide by passing the aqueous phase of the reaction mixture through a column of Sephadex G-15. Figure 13 shows the elution profile obtained from this separation. Quantitation of
Fig. 12. Inhibition of precipitation with OFd and its homologous antiserum in the presence of varying amounts of the octapeptide.
Fig. 13. The elution profile of the NH$_2$-acetylated octapeptide. The first peak contained the peptide, and the second $^{14}$C-acetic acid.
peptide after elution, washing and drying yielded a preparation containing $3.5 \times 10^6$ cpm/micromole.

In order to increase the titer of the antibody preparation used for equilibrium dialysis measurements and to increase the specificity of the reaction, specific antibodies were isolated from the antisera directed against native ferredoxin and OFd. The specific immunoadsorption method of Robbins et al. (1967) was used, in which the antigen is covalently bound to an insoluble support; in this case bromoacetyl cellulose. Specific immunoadsorption was used because it permits the removal of antibodies from serum which will not precipitate with homologous antigen, as in the case of native Fd antiserum. The preparation of the immunoadsorbent involves two steps. First, bromoacetyl bromide is covalently bonded to the bromoacetyl cellulose by an ethereal link, then the antigen is bonded to the bromoacetyl cellulose by an amide link through the free amino groups present on the protein. One of the problems encountered in this technique is the blocking of antibody binding sites by the attachment of the protein to the cellulose; the method thus requires that the binding points on the protein be randomly distributed in order to ensure adsorption of all types of antibody. The ferredoxin molecule presents a special case since, aside from the NH$_2$-terminal amino acid, only one other free amino group occurs in the molecule, at Lys$^3$. Therefore, the points of attachment are limited to one end of the protein, leaving virtually the entire molecule free for antibody binding.
(unless, of course, any antibody was directed to the NH₂-terminus of the protein). Since the antibody directed against the COOH-terminus was to be measured, this was not considered a serious drawback.

Native ferredoxin antiserum and OFd antiserum were adsorbed to an OFd-cellulose conjugate, since, as complement fixation data show, the antiserum to native ferredoxin reacts preferentially with a denatured antigen. After elution of the adsorbed antibody, both preparations were brought to 15.0 ml, and the complement fixation titers determined. The purified preparations titered at 1/160 and 1/900 for native ferredoxin antiserum and OFd antiserum, respectively. When compared to the original titers of 1/40 and 1/200 this represents a recovery of 83.3% and 90%, respectively, which are within the range of 80-90% recovery reported by Robbins et al. (1967). The concentrations of protein were 420 µg/ml for OFd antibody and 240 µg/ml for ferredoxin antibody. It is interesting to note that while there was a five-fold difference in titre between the purified preparations of OFd and native Fd antibody, there was a less than two-fold difference in the protein concentration. This may reflect either a decreased avidity in the antibody population of Fd antiserum, or that a large part of the antibody population of Fd antiserum is directed to a derivative other than native ferredoxin. This question cannot be settled until the fate of native ferredoxin \textit{in vivo} has been established, although from cross-reaction data it appears that
antibodies are preferentially directed against a denatured, ligand-free derivative.

The equilibrium dialysis method of determining reaction between hapten and antibody may be considered the best available, since it measures direct binding, whereas other methods, such as inhibition of precipitation and of complement fixation, measure a negative quantity, allowing greater interference due to non-specific effects. It is also a sound way to measure the association constant of the interaction between the hapten and the antibody binding site since the measurements are made at equilibrium. The following equation expresses the interaction between a low molecular weight hapten and its antibody and the derivation of the association constant:

\[ H + B = HB \]

\[ \frac{(HB)}{(H)(B)} = K_0 \]

The molar concentration of antibody and bound and free hapten must be determined in order to establish \( K_0 \). The amount of free hapten can be determined at equilibrium by doubling the concentration in a given volume found on the antibody-free side of the dialysis membrane, and by subtracting this quantity from the concentration of hapten in the same volume originally added, the quantity of the bound hapten in a given volume of antibody solution, can be determined. Expressed on a molar basis, it is possible to calculate the ratio of the number of moles of hapten bound per mole antibody and the
concentration of free hapten.

In the experiment performed here, labelled hapten at two concentrations, (0.001 and 0.002 micromoles/ml) was dialysed against purified antibodies elicited against OFd and native Fd, and against non-specific gamma globulin and PBS, for 4 days at 4 C, at which time equilibrium was reached.

The results from the equilibrium dialysis experiment are given in Table 4. By the criterion stated above (excess hapten associated with the antibody solution at equilibrium), the octapeptide binds with both OFd and native Fd antisera. While the number of excess counts are low, they are consistently higher than free hapten, and greater than that associated with non-specific gamma globulin. Since no effort was taken to separate labelled from unlabelled peptide, and since more than three concentrations of hapten are required, the $K_o$ and percentage of antibody involved in hapten binding were not calculated.

Binding was demonstrated between the octapeptide and both antisera. This, along with the data obtained from hapten inhibition tests, indicates that the COOH-terminal octapeptide of _C. pasteurianum_ ferredoxin is an area of antigenicity on the protein. It is interesting to note that though OFd and native ferredoxin antisera were evoked by chemically quite different antigens, both contained antibodies directed against the same portion of the protein. This observation is interesting in the light of the results obtained by Gerwing and Thompson (1969) on reduced and carboxymethylated lysozyme. They
<table>
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<td></td>
<td>0.001 mc.mole/ml.</td>
<td>0.002 mc.mole/ml.</td>
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<tr>
<td></td>
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<td>Outside</td>
<td>Difference</td>
<td>Inside</td>
<td>Outside</td>
<td>Difference</td>
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<td>38</td>
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<td>1150</td>
<td>62</td>
</tr>
<tr>
<td>OFd Antiserum</td>
<td>694</td>
<td>639</td>
<td>55</td>
<td>1360</td>
<td>1255</td>
<td>105</td>
</tr>
<tr>
<td>Gamma globulin</td>
<td>673</td>
<td>664</td>
<td>9</td>
<td>1295</td>
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</table>

Table 4. Equilibrium dialysis data.
found that a peptide possessing haptenic activity involved the area of the protein found by Shinka et al. (1967) to be haptenic with respect to native lysozyme and its antiserum, and included the NH$_2$-terminal portion of the "loop peptide" studied by Arnon and Sela (1969).

The results reported here indicate that there are areas of antigenicity on *C. pasteurianum* ferredoxin which do not directly involve participation of cysteine residues, and that the COOH-terminal octapeptide is one of these. It would be interesting to determine the species against which antibodies are produced when native ferredoxin is used as an antigen, and further studies will involve testing of other areas of the molecule for their ability to bind antibody directed against the whole molecule, and to establish the minimum sequence of amino acids within the COOH-terminal octapeptide which is capable of binding antibody directed against the whole molecule.
CONCLUDING REMARKS

The work described in this thesis involved a study of the antigenic characteristics of the electron transport protein, ferredoxin, isolated from *Clostridium pasteurianum*. Four main conclusions can be drawn from the data compiled in this thesis:

(1) Ferredoxin and its performic acid oxidized derivative are antigenic in rabbits as measured by the complement fixation test. The oxidized derivative appears to be a better antigen and evokes the production of precipitating antibody. This conclusion, however, is tentative, since only one animal was used for each immunization. In the case of performic acid oxidized ferredoxin antiserum, cross-reaction was also observed by the quantitative precipitation method.

(2) Cysteine is not critically involved in the amino acid sequences of at least two of the antigenic regions of the ferredoxin molecule, since cross-reaction, as measured by complement fixation, was observed in all instances between native ferredoxin antiserum and performic acid oxidized ferredoxin antiserum with four forms of ferredoxin. Each form contained different cysteine residues; native ferredoxin, apoferredoxin, performic acid oxidized ferredoxin and carboxyamidomethylated ferredoxin.
(3) Antibodies produced by inoculation of an adjuvant preparation of native ferredoxin appear to be directed against a modified species of the protein. Ferredoxin is probably denatured and freed of ligands in vivo before stimulating an antibody response, since the antiserum produced reacted to a greater degree with apoferredoxin and carboxamidomethylated ferredoxin than with its supposedly homologous antigen, as measured by complement fixation. Both apoferredoxin and carboxamidomethylated ferredoxin are denatured and free of iron and hydrogen sulfide.

(4) The COOH-terminal octapeptide is an area of antigenicity in both native ferredoxin and its performic acid oxidized derivative as measured by inhibition of complement fixation and precipitation, and by direct binding studies using the equilibrium dialysis method.


