Location of Disulphide Bridges by Diagonal Paper Electrophoresis

THE DISULPHIDE BRIDGES OF BOVINE CHYMOTRYPSINOGEN A

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1. A new method is described for 'fingerprinting' cysteic acid peptides derived from the disulphide bridges of proteins. Cystine peptides are separated by paper electrophoresis and oxidized on paper by performic acid vapour. Electrophoresis at right angles to the first direction produces parallel groups of cysteic acid peptides lying off a diagonal. This 'fingerprint' reveals the way in which the cysteic acid peptides were originally joined in the protein. 2. The method allows a very easy selective purification of cysteic acid peptides. 3. By applying this method to bovine chymotrypsinogen A, we found that the half-cystine residues were linked 1-122, 42-58, 136-201, 168-182 and 191-220.

Bovine chymotrypsinogen A and its derivative enzymes, especially α -chymotrypsin, have been the object of extensive studies of structure and function and these studies have been frequently reviewed (Niemann, 1964; Keil, 1965; Bender & Kézdy, 1965). The positions of the five disulphide bridges of chymotrypsinogen reported previously (Brown & Hartley, 1963) and in this paper, together with the complete amino acid sequence of 245 residues (Hartley, 1965), completes the covalent structure. Independent studies of the disulphide bridges (Keil, Prusik & Šorm, 1963) are in agreement with those reported here. When the amino acid sequence being determined independently by Keil and co-workers (Keil, 1965) is complete and differences with that of Hartley (1964) resolved, the structure may be considered as established. This information may be used to assist the elucidation of the three-dimensional structures of chymotrypsinogen and its enzymes by X-ray crystallography that is being pursued in several Laboratories (reviewed by Kraut, 1965).

In 1963, when we began this attempt to determine how the ten half-cystine residues of chymotrypsinogen were linked, the only precedents available to us were the studies of Ryle, Sanger, Smith & Kitai (1955) on the three disulphide bridges of insulin, and of Ryle & Anfinsen (1957) and Spackman, Stein & Moore (1960) on the four bridges of ribonuclease. These pioneer workers purified cystine peptides from partial acid or

enzymic digests of the proteins, which they then oxidized to pairs of cysteic acid peptides. Amino acid analysis of the purified cysteic acid peptides was sufficient to identify the corresponding bridges, since all or most of the amino acid sequence was known.

Although at the time we knew most of the sequence of chymotrypsinogen, we were afraid that this approach would be a formidable undertaking, since chymotrypsinogen (245 residues) is much bigger than insulin (51 residues) or ribonuclease (124 residues).

We therefore developed a method based on a 'diagonal' paper-electrophoretic technique. We found this to be a powerful method and consider that it is of interest, not only for determining the positions of disulphide bridges in proteins of known sequence, but as a general method for examining sequences around half-cystine residues of proteins of unknown structure. Indeed, since our preliminary report (Brown & Hartley, 1963) this method has been applied to studies of the disulphide bridges of Bence-Jones protein (Milstein, 1964*a*), bovine chymotrypsinogen B (Smillie & Hartley, 1965), hen's-egg lysozyme (Brown, 1964), bovine trypsinogen (Kauffman, 1965) and porcine elastase (Hartley, Brown, Kauffman & Smillie, 1965; Smillie & Hartley, 1964*a*), and to studies of the active centre of chymotrypsin (Smillie & Hartley, 1964*b*; Brown & Hartley, 1964).

The present paper has the dual purpose of

describing the diagonal method in detail and of presenting the evidence for the arrangement of the five disulphide bridges of chymotrypsinogen A.

MATERIALS AND METHODS

Diagonal electrophoresis. (a) General principle. The general principle of diagonal electrophoresis involves the following three steps:

(1) Separation of a complex peptide mixture by electrophoresis.

(2) Treatment of the electrophoretogram so as to cause a specific modification of some components that will effect a change in charge or molecular weight.

(3) A second electrophoresis at right angles to the first.

Components unaffected by the treatment in step (2) will have the same mobility in the second dimension of electrophoresis and will therefore lie on a 45° diagonal line, whereas components modified in charge or molecular weight will have a different mobility in the second electrophoresis and will therefore lie off the diagonal. The method that we describe is based on the conversion of cystine peptides into pairs of cysteic acid peptides by exposure of the paper to performic acid vapour, thus creating a negative charge with each cysteic acid residue produced.

Diagonal electrophoretic techniques have previously been used for many diverse purposes. A diagonal electrophoretic and chromatographic method involving performic acid oxidation of cystine, methionine and glutathione was used by Mikeš & Holejšovský (1957) to identify these components in amino acid mixtures. Milstein & Sanger (1961) describe a method by which histidine peptides are revealed in a diagonal electrophoretic 'map' after photo-oxidation. Naughton & Hagopian (1962) used diagonal 'maps' of peptides from trypsin digests treated on paper with carboxypeptidase B to reveal the peptide deriving from the C-terminus of proteins, the latter being the only peptide remaining on the diagonal. Milstein (1964b) employed diagonal electrophoresis to study the interconversion of α - and β -aspartyl peptides produced during partial acid hydrolysis of phosphoglucomutase. It is clear that diagonal techniques can be widely exploited.

(b) Diagonal method for cystine peptides. The essential steps of the diagonal technique for determining the positions of disulphide bridges in proteins are outlined below, and their specific application to chymotrypsinogen A is discussed in detail in subsequent sections.

(1) The protein is digested with pepsin or other suitable enzyme.

(2) The peptides are subjected to electrophoresis as a band on paper, preferably at pH 6.5.

(3) A marker strip, cut from this preparative electrophoretogram, is exposed to the vapour of performic acid.

(4) The oxidized strip is stitched to a sheet of paper and electrophoresis carried out at pH 6.5 perpendicular to the strip.

(5) The diagonal 'fingerprint' is developed with ninhydrin or other detection reagents. This reveals the position of cystine peptides in the preparative electrophoretogram.

(6) The bands of cystine peptides are cut out from the paper of step (2) and oxidized with performic acid vapour.

(7) The oxidized bands are stitched to sheets of paper and subjected to electrophoresis perpendicular to the bands.

(8) The bands of cysteic acid peptides are located by developing guide strips with ninhydrin. The diagonal 'fingerprint' indicates which bands are related. Contaminants remain in the 'diagonal position'.

(9) The purified cysteic acid peptides are eluted for composition and sequence analysis.

Enzymes. Bovine chymotrypsinogen A (crystallized, salt-free; batch C.G.541), pepsin (twice-crystallized; batch PM692) and trypsin (twice-crystallized, salt-free; batch TRSF813-4) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin B was a preparation of 'bacterial trypsin' (Hunt & Ottesen, 1956) from Novo Terapeutisk Laboratorium, Copenhagen, Denmark.

Peptic digestion of chymotrypsinogen. In a typical experiment, 100 mg. of chymotrypsinogen and 10 mg. of pepsin are dissolved in 20 ml. of 0.05 N-HCl and incubated at 37° for 18 hr. with occasional stirring. Such high enzyme/substrate ratios are desirable to minimize the amount of insoluble 'core'. Occasionally digestions were carried out in 5% (v/v) formic acid with essentially similar results. One might fear that cysteic acid peptides derived from the autolysis of pepsin would appear in the final diagonal 'fingerprint', but control experiments with pepsin alone at corresponding concentrations showed that such artifacts, although possibly present, were undetectable.

Paper electrophoresis of the digest at pH 6.5. Internal fluorescent markers are added to the digest before electrophoresis, to calibrate the mobility of peptides and to act as guides for eluting bands in preparative runs. A useful mixture can conveniently be made by treating 0.1 M-arginine in 1.0 M-NaHCO₃ with an equal volume of 0.2 M-DNS* chloride (British Drug Houses Ltd., Poole, Dorset) in acetone. After 5 hr. at 37° the mixture is diluted 1000-fold to give approx. 0.1 mM-DNS-Arg and 0.1 mM-DNS-OH. At pH 6.5, the yellow-fluorescent DNS-Arg is neutral, whereas the blue-fluorescent DNS-OH moves with 63% of the mobility of aspartic acid. At pH 2.0, DNS-OH is neutral whereas DNS-Arg moves with the same mobility as glycine.

This fluorescent marker (0.05 ml.) is added to 20 ml. of the digest, and the whole applied directly as three 35 cm. bands on full sheets of Whatman 3MM paper by using a pipette with a capillary tip bent at 90° angle. The resulting wet band is about 7 cm. wide and contains approx. 1 mg. of digested protein/cm. length. This method of application avoids the precipitation of peptides that frequently occurs during evaporation or freeze-drying of digests, and these precipitated peptides are often difficult to redissolve. Moreover, by this method any insoluble peptides that are present are distributed over a wide area, and no longer form an impenetrable barrier at the origin (a frequent cause of distortions in paper electrophoresis).

Amino acid markers are then applied as spots alongside the bands of digest, and the papers dried in a current of cool air. Soft folds are made along and parallel to the origin, so that, when placed on a glass plate for wetting with buffer, the wide band of peptides lies in an inverted V out of contact with the plate. The pH 6.5 buffer (pyridine-acetic acid-

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; DNS-OH, 1-dimethylaminonaphthalene-5-sulphonic acid; in amino acid sequences, CySO₃H, MetSO₂, Asn and Gln refer to cysteic acid, methionine sulphone, asparagine and glutamine residues respectively.

water, 25:1:225, by vol.) is carefully streaked on either side of the band and allowed to soak up to the apex of the inverted V. Excess of buffer is blotted away and electrophoresis carried out at 3000 v (60/cm.) for 50 min. in a Michl (1951) apparatus with toluene as coolant.

The papers are dried in cool air and the fluorescent bands outlined in pencil. Marker strips can be developed by cadmium-ninhydrin reagent (Heilmann, Barollier & Watzke, 1957) or by other colorimetric reactions.

Performic acid oxidation of the diagonal strip. A strip about 3 cm. wide is cut from the edge of the sheet and incubated for 2 hr. at room temperature in a desiccator containing a freshly prepared mixture of 20 ml. of 98% (v/v) formic acid and 1 ml. of 30% (v/v) H_2O_2 in a Petri dish. A convenient support for the strips is a spiral glass rod with 3 mm. $\times$ 20 mm. rods fused at right angles at 3 cm. intervals, and a 9 mm. $\times$ 10 cm. rod at the centre that acts as a handle. Care was taken to avoid contamination of the paper or desiccator by halides, as halogens may be generated that react with histidine, tyrosine and phenylalanine.

Preparing the diagonal 'fingerprint'. The oxidized strip is removed from the desiccator and left in a fume cupboard for at least 30 min. to allow evaporation of the formic acid and performic acid. Then it is stitched across the centre of a full sheet of Whatman 3MM paper with a zigzag stitch with a sewing machine, and the paper behind the strip cut out, care being taken not to cut any threads. A streak of fluorescent marker may be applied along the strip, to assist in calibrating mobilities. After wetting with pH 6.5 buffer, electrophoresis is carried out as before at 3000 v for 50 min.

Identification of peptides on paper. When dry, the paper is examined under an ultraviolet lamp and then dipped in cadmium-ninhydrin reagent (Heilmann *et al.* 1957). The colour and rate of development of the spots during incubation at room temperature is often characteristic of a particular peptide. Thus we found that peptides with *N*-terminal cysteic acid gave yellow spots after 1 hr. that turned red overnight, whereas the yellow spots given by *N*-terminal glycine were stable for at least a week. *N*-Terminal valine or isoleucine gave red colours that needed several days for maximum development.

Separation of neutral cystine peptides. The neutral band at pH 6.5 will often contain a mixture of cystine peptides, and it is therefore dangerous to assume that cysteic acid peptides arising from this band are necessarily related. This difficulty is removed by cutting out the neutral band before oxidation, stitching it to another sheet of similar paper and re-running a paper electrophoresis at another pH, e.g. pH 2 or pH 3.5. Strips from this can then be oxidized and re-run at the same pH so that new pH 2 diagonals or pH 3.5 diagonals of the neutral cystine peptides will arise. However, an alternative procedure can be adopted with these neutral cystine peptides: the oxidized strip from the pH 2 or pH 3.5 electrophoresis can be re-run at right angles at pH 6.5, whereupon the cysteic acid pairs move to the anode while the neutral peptides are left in a band at the origin.

pH 2 or pH 3.5 diagonal 'fingerprints'. Diagonal 'fingerprints' at pH 2 or pH 3.5 have the disadvantage that cystine peptides tend to 'tail' in the first dimension, and it is less easy to interpret the charge pattern of the resulting cysteic acid peptides. Nevertheless, pH 2 diagonals are useful for locating peptides containing cysteic acid and a basic residue, since these are neutral at pH 6.5 but cationic

at pH 2, whereas other cysteic acid peptides are anionic at pH 6.5 and neutral at pH 2. Diagonals at pH 3.5 can also be of advantage, since almost all cystine peptides tend to be either cationic or anionic at this pH.

Isolation of cysteic acid peptides. If a very dilute ninhydrin detection spray were used, it would be possible to cut out and elute the cysteic acid peptides directly from a diagonal 'fingerprint'. However, we have preferred to use the diagonal 'fingerprint' as a key to a simple preparative isolation of the cysteic acid peptides. For example, the diagonal 'fingerprint' of a peptic digest of chymotrypsinogen (shown in Fig. 1) shows four major groups of cysteic acid peptides arising from cystine peptides originally present in bands A-D in the first electrophoresis. These bands may be cut out from the original paper electrophoresis and oxidized with performic acid vapour as above. Each band is then stitched across a full length of Whatman 3MM paper, a streak of fluorescent marker solution is applied along the band and electrophoresis at pH 6.5 is carried out at right angles to the band. When the electrophoretogram is dry, the fluorescence is examined, and 1 cm. guide strips are developed with cadmium-ninhydrin reagent. The mobilities of the cysteic acid peptides relative to the fluorescent marker serve to relate them to the original diagonal 'map', and the fluorescent bands also provide a useful guide in cutting out the bands of peptides for elution, especially where any distortions of the bands have occurred during electrophoresis.

In this way, the cysteic acid peptides may frequently be purified in a single operation, since they are automatically selected from all other peptides, which remain in the original 'diagonal position'. Should further purification be necessary, the band of cysteic acid peptide can be submitted to electrophoresis at another pH. This is particularly desirable for peptides that are neutral at pH 6.5, since they are frequently mixtures, and electrophoresis at pH 3.5 is very useful for this purpose.

Further digestion of the bands of cystine peptides. Occasionally the sequence between two half-cystine residues may be resistant to splitting by pepsin and, unless these form an internal loop, peptides with two bridges connecting three chains may be found in the digest. In such cases the pairing of the cysteic acid peptides will be ambiguous, and it is necessary to break the chain between the bridges and separate the resulting cystine peptides. Also, in other cases, where digestion with pepsin has been notably unspecific or where two cystine peptides lie in the same band, a subsequent digestion with another enzyme may simplify the diagonal pattern and assist in its interpretation.

The appropriate band is therefore eluted and redigested with another enzyme. Conditions that might favour disulphide interchange, such as high pH or presence of thiols, were avoided. We have generally used trypsin, chymotrypsin or subtilisin at pH 6.5, well below their pH optima, but some studies of chymotryptic digests in 0.5% NH_4HCO_3 at pH 8 gave no suggestion of disulphide interchange. We suspect that cystine peptides are peculiarly resistant to proteolysis and have tended to use high enzyme/substrate ratios (e.g. 1:10 rather than 1:100) for this reason.

Amino acid analysis. Peptides were hydrolysed in 5.7 N-HCl in sealed evacuated tubes, normally for 18 hr. at 105°. A Beckman-Spinco model 120 amino acid analyser was generally used with the accelerated flow system of Spackman

(1963) or modifications thereof. Occasional analyses were performed after two-dimensional separations of amino acids on paper, by dipping in cadmium-ninhydrin reagent (Heilmann *et al.* 1957), developing the pink spots at 60° for 15 min. and eluting these in 95% (v/v) ethanol (L. F. Smith, personal communication). By reading the extinction at 510 m μ an accuracy of $\pm 15\%$ can be expected from about 0.01 μ mole of amino acid.

RESULTS

Diagonal 'fingerprints' of the peptic digest of chymotrypsinogen. Fig. 1 is a drawing of a pH 6.5 diagonal 'fingerprint' of a peptic digest of chymotrypsinogen and illustrates the nomenclature of the cysteine acid peptides that lie off this diagonal. There are four main groups of these derived from cystine peptides in bands 1A–1D in the original electrophoresis. A close study of this pattern gives some clues about the nature of the cystine peptides.

Band 1A, for example, must contain anionic

cysteine peptides that give rise to peptides 1A1–1A3 after oxidation. The mobility of peptides 1A2 and 1A3 suggests that they contain one or probably two acidic groups as well as cysteine acid, whereas cysteine acid is probably the only acidic residue in peptide 1A1. The cadmium-ninhydrin colour of peptide 1A1 was yellow becoming red after 24 hr., peptide 1A2 gave a rapid red colour and peptide 1A3 gave a red colour that developed very slowly. An intelligent guess for this bridge would be a structure of the form:

where full stops indicate known order, commas indicate unknown order, — indicates a neutral

Fig. 1. pH 6.5 diagonal 'map' of a peptic digest of chymotrypsinogen. Conditions were as described in the text. The major cysteine acid peptides are hatched. DNS-OH indicates the position of the internal fluorescent marker. The electrophoretic mobilities of the peptides are shown relative to that of DNS-OH (-1.0).

residue, 'Asp' stands for aspartic acid or glutamic acid and 'Val' for valine or isoleucine.

Although it is normally dangerous to conclude that cysteine peptides in the neutral band are necessarily from the same bridge, since peptides 1B1 and 1B2 are the only major spots we may tentatively assume that they are related. Both give a positive Pauly reaction (Dent, 1947) and therefore probably contain histidine. Hence an additional acidic residue must be postulated to explain why the cystine peptide is neutral. A structure of the following form is possible:

Band 1C must consist almost entirely of slightly cationic cystine peptides since no ninhydrin colour remains in the diagonal position after oxidation. Since all three oxidation products are anionic at pH 6.5, a double cystine peptide of the form:

might be indicated, where 'Lys' indicates lysine or arginine, or alternatively a histidine peptide such as:

For band 1D, several structures are possible, but one such as:

might be one component.

Such very tenuous arguments can seldom be relied on, but are very useful in designing purification procedures for isolating the cysteine acid peptides, and occasionally allow one to guess the pattern of the disulphide bridges from the diagonal 'fingerprint' alone.

Analysis of band 1A. The cysteine acid peptides from this band are not well resolved at pH 6.5 (Fig. 1): peptide 1A1 lies close to the diagonal and peptides 1A2 and 1A3 have very similar mobilities. The strips containing band 1A were therefore oxidized and subjected to electrophoresis at pH 3.5. Under these conditions, peptide 1A1 separated well but peptides 1A2 and 1A3 were still slightly cross-contaminated. Table 1 shows the amino acid analyses of these peptides, which according to the sequence of Hartley (1964) could only derive from the bridge:

In fact, the composition of peptide 1A1 prompted a reinvestigation of the sequence of the A-chain of α -chymotrypsin (W. R. Gray & B. S. Hartley, unpublished work) that corrected errors in the sequence given by Meedom (1958). Cross-contamination of peptides 1A2 and 1A3 results in non-integral values for alanine in Table 1, but the electrophoretic mobility and cadmium-ninhydrin colour of these peptides support the structures ascribed above. Hence CyS-I is linked to CyS-IV in chymotrypsinogen.

Analysis of band 1B. The neutral band 1B was submitted to electrophoresis at pH 3.5 to resolve any possible mixtures of cystine peptides. A single Pauly-positive band was located: this was the most cationic peptide at this pH. When oxidized and run again at pH 6.5 this gave peptides 1B1 and 1B2 (Fig. 1), which were eluted. Amino acid analysis (Table 1) showed that these peptides must derive from the bridge:

Hence CyS-II and CyS-III form a loop that brings His-40 close to His-57 in the primary structure.

Analysis of band 1C. The diagonal (Fig. 1) suggests that component 1C may be a pure cystine peptide, since no ninhydrin colour remains in the 'diagonal position'. A portion of this band was eluted, hydrolysed and analysed (Table 1). The rest of the band was oxidized on paper, giving the cysteine acid peptides 1C1, 1C2 and 1C3 after electrophoresis at pH 6.5. Component 1C itself and peptides 1C1 and 1C2 are fluorescent bands, suggesting that tryptophan could be present, but peptide 1C3 shows no trace of fluorescence. Preliminary analyses of peptide 1C1 indicated that it contained both the CyS-VIII and the CyS-IX sequence, whereas peptide 1C2 was derived from CyS-X and peptide 1C3 from CyS-V. The peptides were appreciably cross-contaminated, however, and so an alternative purification was made by running

Table 1. *Peptic peptides from chymotrypsinogen (Fig. 1)*

Amino acid compositions were obtained by ion-exchange techniques except where stated otherwise (peptide 1A2); ratios are uncorrected for hydrolysis time. Electrophoretic mobilities are given relative to that of DNS-OH (−1.0). The Pauly spray reaction was carried out as described by Dent (1947); relative intensities of the orange-red histidine colour are shown (+) and the purple-red tyrosine colour is indicated (Tyr).

Peptide	Amino acid composition (ratios)	Electrophoretic mobility at pH 6.5	Cadmium- ninhydrin colour	Other details
1A1	CySO ₃ H, Glu, Pro, Gly, Ala, Val, Ile 0.8 1.1 0.9 1.0 1.0 1.0 1.0	−0.68	Yellow→red	
1A2	CySO ₃ H, Asp, Ser, Pro, Ala, Val, Leu 0.9 1.9 2.1 + 1.7 1.0 1.1	−1.07	Red	Paper quantitation
1A3	CySO ₃ H, Asp, Ser, Pro, Ala, Val, Leu 1.0 2.0 1.9 1.1 1.2 1.0 1.1	−1.14	Slow red	
1B1	CySO ₃ H, Ser, Gly, Leu, Phe, His, (Ala) 1.0 0.9 2.0 1.0 1.0 0.7 (0.2)	−0.40	Orange	Pauly (+)
1B2	CySO ₃ H, Asp, Thr, Ser, Gly, Ala, Val, His 1.0 1.0 1.8 1.1 1.3 1.9 1.0 1.0	−0.51	Red	Pauly (3+)
1C	CySO ₃ H, Asp, MetSO ₂ , Thr, Ser, Pro, Gly, Ala, Val, Leu, Tyr, Lys 4.0 2.4 0.9 6.1 4.9 2.0 8.1 2.9 3.2 1.5 0.6 2.0	+0.22	Brown	Pauly (+); Ehrlich (+); fluorescent
1C1	CySO ₃ H, Asp, MetSO ₂ , Ser, Pro, Gly, Ala, Val, Leu, Lys, (Thr) 1.8 2.0 0.9 2.1 1.1 4.1 1.0 1.0 1.0 1.8 (0.5)	−0.31	Orange	Fluorescent
1C2 + '1C4'*	CySO ₃ H, Thr, Ser, Pro, Gly, Ala, Val, Leu, Tyr 1.4 4.7 3.3 0.9 3.4 1.2 1.3 0.4 0.4	−0.42	Yellow	Fluorescent; Pauly (Tyr)
1C3	CySO ₃ H, Thr, Gly, Ala, Val 1.0 3.8 1.9 1.9 1.0	−0.51	Red	
1D2	CySO ₃ H, Asp, MetSO ₂ , Thr, Ser, Gly, Ala, Val, Ile, Leu, Tyr, Lys 1.6 3.0 1.0 2.0 3.1 3.1 3.1 0.8 1.6 0.5 0.8 3.7	+0.24	Red	Fluorescent; Pauly (Tyr)
1D3	CySO ₃ H, Ser, Gly, Ala, Val, Ile 0.9 1.7 2.0 2.0 0.9 0.8	−0.57	Slow red	
1D4	CySO ₃ H, Ser, Gly, Ala, Ile, (Thr) 0.9 1.8 1.8 2.3 0.9 (0.3)	−0.70	Slow red	
1D5	CySO ₃ H, Ala, Ile, (Gly) 0.8 1.1 0.8 (0.3)	−0.93	Slow red	

* Probably a mixture of peptide 1C2 (CySO₃H, Thr₃, Ser₄, Pro, Gly₂, Val, Tyr) plus approx. 50% of peptide '1C4' (CySO₃H, Thr₄, Gly₂, Ala₂, Val, Leu, Trp).

the band of oxidized component 1C by electrophoresis at pH 2, whereupon peptide 1C1 gave a cationic band that stained orange with cadmium-ninhydrin reagent, whereas peptides 1C2 and 1C3 were neutral. When this neutral band was re-run at pH 6.5 (100 min., 60 v/cm.) peptide 1C2 gave a fluorescent band with a yellow ninhydrin colour and peptide 1C3 separated with a pink ninhydrin colour and was not fluorescent.

The amino acid analyses of these peptides are shown in Table 1. The analysis of peptide 1C2 is clearly equivocal. We believe that it is a mixture of peptide 1C2 (residues 216–228) together with a 45% contamination with peptide '1C4' (residues 131–143), which is related to peptide 1C3. The analysis and electrophoretic mobility are consistent with such an hypothesis. Peptide 1C1 can be identified more definitely with residues 190–207 and peptide 1C3 with residues 131–140, as shown in Fig. 3. There is little doubt that component 1C

is a cystine peptide with two disulphide bridges formed by CyS-V, CyS-VIII, CyS-IX and CyS-X.

Subtilisin digestion of band 1C. To resolve the ambiguity in interpreting these disulphide bridges, it is clearly necessary to split component 1C between the two bridges and to separate the resulting cystine peptides. For this purpose we chose digestion by subtilisin.

A portion of band 1C, corresponding to the yield from about 0.5 μmole of chymotrypsinogen, was eluted with 1 ml. of pyridine-acetate buffer, pH 6.5. Subtilisin (50 μg.) was added and the mixture incubated for 16 hr. at room temperature. After the addition of fluorescent marker mixture, the digest was applied as a 30 cm. band on Whatman no. 1 paper for electrophoresis at pH 6.5 (40 min., 60 v/cm.). Oxidation of a strip from this paper gave the diagonal 'fingerprint' shown in Fig. 2. Four major bands of cystine peptides, 2A–2D, were found, and the cysteic acid peptides from these

Fig. 2. pH 6.5 diagonal 'map' of a subtilisin digest of cystine peptides eluted from band 1C (Fig. 1). Conditions were as described in the text. Peptides containing cysteic acid are hatched. The electrophoretic mobilities of the peptides are shown relative to that of DNS-OH (-1.0).

were isolated in the same manner as described above.

Band 2A, the most anionic, gave two cysteic acid peptides 2A2 and 2A3 and left a single peptide, 2A1, on the diagonal. All of these were yellow with cadmium-ninhydrin reagent, and their compositions are shown in Table 2. Peptide 2A2 (CySO₃H,Thr,Ser) clearly arises from CyS-X, whereas its mate, peptide 2A3 (Ser,CySO₃H, MetSO₂,Gly,Asp,Ser), must come from CyS-VIII, showing that CyS-VIII and CyS-X are linked in chymotrypsinogen. At this stage, the pattern of disulphide bridges in the protein may be inferred, since the remaining bridge in peptide 1C must be V-IX, and I-IV and II-III have already been determined. The missing bridge must therefore be VI-VII.

Peptide 2A1, although not a cysteic acid peptide, is noteworthy since it represents a piece of sequence Gly-Asp-Ser-Gly-Gly-Pro-Leu that is split out by subtilisin between CyS-VIII and CyS-IX, and includes the active-centre Ser-195 of chymotrypsin.

Band 2B must contain a slightly anionic cystine peptide, which gives peptides 2B1 (Ser,CySO₃H,-

MetSO₂) and 2B2 (CySO₃H,Thr,Ser) after oxidation. Although this cystine peptide contains no acidic or basic amino acid, its slightly anionic mobility is consistent with an *N*-terminal half-cystine, where the rather low p*K* results in a partial net negative charge at pH 6.5 (Ryle *et al.* 1955). Peptide 2B2 appears to be identical with peptide 2A2, whereas peptide 2B1 is a smaller relative of peptide 2A1, and so these peptides confirm the bridge CyS-VIII to CyS-X.

The pH 6.5 diagonal (Fig. 2) shows that band 2C gives rise to a neutral peptide, 2C1, and anionic peptides 2C2 and 2C3. Because peptide 2C1 is neutral at pH 6.5 and could be a mixture, preparative electrophoresis of the cysteic acid peptides was carried out at pH 3.5, giving peptide 2C1 (Val,CySO₃H,Lys), which must derive from CyS-IX, and peptides 2C2 (CySO₃H,Val,Thr,Thr,Gly) and 2C3 (CySO₃H,Val,Thr) from CyS-V. Hence the bridge CyS-V to CyS-IX is proved.

The most cationic cystine peptide in the subtilisin digest, 2D, gave cysteic acid peptides 2D2 and 2D3 that are identical with peptides 2C2 and 2C3 in composition, electrophoretic mobility and

Table 2. *Subtilisin peptides from band 1C (Fig. 2)*

Amino acid compositions were obtained by ion-exchange techniques except where stated otherwise (peptides 2A2, 2C1 and 2C3); ratios are uncorrected for hydrolysis time. Electrophoretic mobilities are given relative to that of DNS-OH (−1.0).

Peptide	Amino acid composition (ratios)	Electrophoretic mobility at pH 6.5	Cadmium-ninhydrin colour	Other details
2A1	Asp, Ser, Pro, Gly, Leu 1.0 1.1 0.8 3.0 1.0	−0.55	Yellow	On the diagonal
2A2	CySO ₃ H, Thr, Ser 0.9 1.0 1.3	−0.94	Yellow	Paper quantitation
2A3	CySO ₃ H, Asp, MetSO ₃ , Ser, Gly 1.1 1.0 0.8 1.8 1.1	−1.11	Yellow	
2B1	CySO ₃ H, MetSO ₃ , Ser 1.0 0.7 1.0	−0.86	Yellow	
2B2	CySO ₃ H, Thr, Ser 0.8 1.0 1.1	−0.94	Yellow	
2C1	CySO ₃ H, Val, Lys + + +	0	Red	Paper qualitative
2C2	CySO ₃ H, Thr, Gly, Val, (Ser) 1.0 1.9 1.0 1.1 (0.5)	−0.75	Yellow→red	
2C3	CySO ₃ H, Thr, Val 0.8 1.1 1.2	−0.92	Yellow→red	Paper quantitation
2D1	CySO ₃ H, Val, Lys 1.0 1.0 1.9	+0.67	Red	On the diagonal
2D2	CySO ₃ H, Thr, Gly, Val 1.0 2.2 0.9 1.0	−0.75	Yellow→red	
2D3	CySO ₃ H, Thr, Val + + +	−0.92	Yellow→red	Paper qualitative

Fig. 3. Structure of cystine peptide 1C and the subtilisin peptides derived from it. Major splits by pepsin (P) or subtilisin (S) are indicated ↓, and minor splits ↓. The peptides are numbered as in Figs. 1 and 2 and Tables 1 and 2, and the amino acid residues as in Fig. 8. The Roman numerals indicate the order in which the half-cystine residues occur along the peptide chain of chymotrypsinogen.

colour with cadmium–ninhydrin reagent (Table 2). The mate to these is the cationic peptide 2D1 (Val,CySO₃H,Lys), which is clearly related to peptide 2C1. Hence peptide 2D is another fragment from the CyS-V to CyS-IX bridge. Our conclusions about the probable sequence of these peptides are summarized in Fig. 3.

Analysis of band 1D (Fig. 1). At this stage of the investigation we had located cysteic acid peptides accounting for eight of the ten half-cystine residues of chymotrypsinogen and it was easy to conclude that the remaining CyS-VI and CyS-VII should be paired and should be found in band D. Indeed, band 1D gave three major anionic peptides, 1D3, 1D4 and 1D5 (Fig. 1 and Table 1), which corresponded to sequences around CyS-VII. However, a major cationic peptide, 1D2, well below the diagonal, which we expected to be the mate to the CyS-VII peptides, had an amino acid composition that was incompatible with the sequence around CyS-VI. We at first assumed that peptide 1D2 might be contaminated with other unrelated cysteic acid peptides, but purification by electrophoresis at pH 3.5 and 1.9 did not change the

amino acid composition. However, this amino acid composition agrees very well with a sequence including both CyS-VI and CyS-VII, the structure of which is given in Fig. 5. Therefore peptide 1D2 must have existed as a cyclic or loop peptide before oxidation, and we believe that the mate to peptides 1D3, 1D4 and 1D5 is peptide 1D1, which is masked within the diagonal. We did indeed find cysteic acid present in hydrolysates from this region of the diagonal but did not attempt purification of the cysteic acid peptide.

Trypsin digestion of band 1D. We could now see that band 1D (Fig. 1) must contain a mixture of cystine peptides derived from the CyS-VI to CyS-VII bridge, resulting from rather unspecific digestion by pepsin. To simplify this pattern, we eluted a 30 cm. strip of the original band 1D, and incubated this in 0.5 ml. of 0.1 M-pyridine-acetate buffer, pH 5.8, containing 0.2 mg. of trypsin at 37° for 16 hr. After the addition of internal fluorescent marker, the entire sample was applied as a 30 cm. band on Whatman no. 1 paper and submitted to electrophoresis at pH 6.5 (50 min., 60 v/cm.). A 2.5 cm. guide strip of this was then oxidized and re-run at

Fig. 4. pH 6.5 diagonal 'map' of a tryptic digest of cystine peptides eluted from band 1D (Fig. 1). Conditions were as described in the text. The cysteic acid peptides are hatched. The electrophoretic mobilities are shown relative to that of DNS-OH (-1.0).

pH 6.5, giving the diagonal 'fingerprint' shown in Fig. 4. Only the neutral band 3A and the cationic band 3B gave rise to cysteic acid peptides, and each of these peptides was isolated in the usual fashion. Table 3 gives the analytical data for these tryptic peptides.

The cationic band, 3B, gave anionic cysteic acid peptides 3B2, 3B3 and 3B4, which are identical in composition and electrophoretic mobility with the original peptic peptides 1D2, 1D3 and 1D4, and

gave the same slowly-developing pink colour with cadmium-ninhydrin reagent consistent with *N*-terminal isoleucine. They clearly derive from CyS-VII. The mate to this series is the neutral peptide 3B1, which was purified by electrophoresis at pH 2. Its composition (Table 3) agrees with the sequence Leu-Ser-Asn-Thr-Asn-CySO₃H-Lys, derived from CyS-VI.

The neutral band, 3A, must contain a related cystine peptide, since the series of cysteic acid

Table 3. *Tryptic peptides from band 1D (Fig. 4)*

Amino acid compositions were obtained by ion-exchange techniques except where stated otherwise (peptides 3B3 and 3B4); ratios are uncorrected for hydrolysis time. Electrophoretic mobilities are given relative to that of DNS-OH (−1.0).

Peptide	Amino acid composition (ratios)	Electrophoretic mobility at pH 6.5	Cadmium- ninhydrin colour	Other details
3A2	CySO ₃ H, Asp, MetSO ₂ , Ser, Gly, Ala, Val, Ile 0.9 1.0 0.8 2.0 2.0 3.0 0.9 0.9	−0.85	Red	
3A3	CySO ₃ H, Asp, MetSO ₂ , Ser, Gly, Ala, Ile 0.9 1.0 0.9 1.0 1.3 3.0 1.0	−0.98	Red	
3A4	CySO ₃ H, Asp, MetSO ₂ , Ala, Ile, (Ser, Gly) 0.7 1.0 0.9 2.3 0.7 (0.3 0.2)	−1.17	Red	
3B1	CySO ₃ H, Asp, Thr, Ser, Leu, Lys, (Gly) 0.9 2.0 1.0 1.0 0.4 1.0 (0.4)	0	Orange	
3B2	CySO ₃ H, Ser, Gly, Ala, Val, Ile 0.7 2.7 2.2 2.0 1.0 0.7	−0.58	Slow red	
3B3	CySO ₃ H, Ser, Gly, Ala, Ile + + + + +	−0.71	Slow red	Paper qualitative
3B4	CySO ₃ H, Ala, Ile + + +	−0.93	Slow red	Paper qualitative

Fig. 5. Structure of cystine peptide 1D and the tryptic peptides derived from it. Sites of cleavage by trypsin (T) are indicated ↑; bonds partially split by pepsin (P) in the original digestion are shown ↓. Peptides are numbered as in Figs. 1 and 4 and Tables 1 and 3, and amino acid residues as in Fig. 8.

peptides 3A2, 3A3 and 3A4 is the same as 3B2, 3B3 and 3B4 except that an additional Asp-Ala-MetSO₂ sequence is present on the *N*-terminus of each. This explains why component 3A is neutral whereas component 3B is cationic. A peptide 3A1, identical with peptide 3B1, is almost certainly present in the neutral band after oxidation, but we did not try to isolate it.

Fig. 5 summarizes our conclusions about the probable sequence of peptic and tryptic peptides derived from band 1D. It is clear that two groups of cystine peptides are found in the tryptic digest. The neutral group, 3A, probably arises from tryptic cleavage within the loop present in the cystine parent of peptide 1D2, whereas the basic group, 3B, arises from peptides corresponding to 1D3, 1D4 and 1D5 in which the bond between Met-180 and Ile-181 had already been split by pepsin. These peptides provide unambiguous evidence for a loop between CyS-VI and CyS-VII.

Minor components in the peptic diagonal. The four main groups of cysteic acid peptides shown in Fig. 1 all arise from the five unique bridges described above. No indication of disulphide interchange was found in any of the peptides that we investigated, although a few minor spots observed in the peptic diagonal were not purified. But even these minor cysteic acid peptides can best be explained as arising from less specific peptic cleavages. For example, a few small spots can be observed in the slightly anionic band of the peptic diagonal shown in Fig. 1. We suspect that these arise from a neutral cystine peptide derived from the I-IV bridge owing to a minor peptic cleavage between CyS-122 and Asp-128. This would give peptide 1A1 plus a pair of peptides analogous to 1A2 and 1A3 in which the *C*-terminal aspartic acid residues were missing. The electrophoretic mobilities and cadmium-ninhydrin colours of these minor spots are perfectly consistent with such an hypothesis.

DISCUSSION

The amino acid sequences around cystine bridges may prove to be of special interest in protein architecture, and our experience suggests that the diagonal technique that we have described will be useful in studying such sequences. A 'fingerprint' of associated sets of cysteic acid peptides can readily be obtained from a small amount of protein, and the method is very flexible in allowing changes in the digestion conditions or methods of peptide separation to obtain the best 'map'. Perhaps the most important feature, however, is that related pairs of cysteic acid peptides can readily be isolated on a preparative scale from complex mixtures of other peptides.

We chose to study peptic digests of chymo-

trypsinogen A since Spackman *et al.* (1960) had shown that disulphide interchange is minimal between pH2 and pH6. Moreover, chymotrypsinogen A is reversibly denatured below pH2 and is digested completely by pepsin to give soluble peptides without any insoluble 'core'. Unfortunately, however, pepsin is not very specific and tends to give families of peptides arising from a single sequence, which complicates the diagonal 'fingerprints'. With other proteins, a different proteolytic enzyme such as trypsin or chymotrypsin may be preferable for the first digestion. We have not, in general, found it desirable to use a mixture of enzymes in the initial digestion, since one tends to obtain predominantly mixtures of neutral cystine peptides that are difficult to separate by electrophoresis. Further, the possibility of redigesting the isolated cystine peptides with a different enzyme is a potent tool in elucidating the bridges.

We believe that pH6.5 diagonals provide the best preliminary study of the digest. The electrophoretic mobilities of the cystine peptides and the derived cysteic acid peptides can give valuable clues about the distribution of charged residues in the vicinity of the bridges. Fig. 6 demonstrates the patterns expected from six common types of cystine peptide. One can see, for example, that spots 5a and 5b in Fig. 6 indicate a symmetrical distribution of two basic residues, whereas if both basic residues lie on one side of the bridge the pattern of spots 6a and 6b will result.

Complexities can, of course, easily arise, and Fig. 7 illustrates some possibilities. An internal cystine bridge gives only a single spot, cf. 7ab. Spots in the neutral band are not necessarily related (spots 8a, 8b, 9a and 9b) and one of the components such as spot 8a or 9a may remain in the diagonal at the origin. Electrophoresis of this neutral band at another pH can resolve this ambiguity. Non-specific cleavage by pepsin often results in a family of peptides from the same bridge, e.g. spots 9a, 9c and 9d, and the same family may appear elsewhere in the diagonal 'map' if the cystine peptides differ in charge, e.g. spots 9a and 9b. Another difficulty arises where pepsin fails to split between two half-cystine residues, as in spot 10a (Fig. 7). Three cysteic acid peptides will appear off the diagonal (spots 10a, 10b and 10c), but it is easy to confuse such a case with that of a simple family of peptic peptides such as spots 9a, 9c and 9d. In such a case, as we have described above, it is necessary to attempt further proteolysis of the cystine peptide to resolve the ambiguity. Despite such difficulties, a careful examination of the diagonal 'map' can provide a useful guide to further studies.

It might be feared that disulphide interchange

Fig. 6. Hypothetical diagonal 'map' produced by some common types of cystine peptides:

1a	—, —, CyS, —, 'Asp', —	2a	—, —, CyS, —
1b	—, CyS, 'Asp', —	2b	—, CyS, 'Asp', —
3a	—, —, CyS, —, —	4a	—, —, CyS, —, 'Lys'
3b	—, CyS, —	4b	—, —, CyS, —, —
5a	—, —, CyS, —, 'Lys'	6a	'Lys', —, CyS, —, 'Lys'
5b	'Lys', —, CyS, —, —	6b	—, CyS, —

would vitiate conclusions about the nature of the cystine bridges in the protein. In this and subsequent studies we have never detected any peptide whose composition or electrophoretic mobility would lead us to suspect such an interchange. Extensive incubation at alkaline pH, or the presence of free thiol groups might, however, cause trouble. But a small amount of random disulphide interchange should produce low yields of a large variety of cystine peptides that might not interfere with the qualitative diagonal 'map'.

As in most paper techniques, the final yields of

purified peptides are not impressive, ranging from about 5% to about 50% of the theoretical. These low yields are due to the low specificity of peptic cleavage, and to the losses associated with adsorption to and elution from paper. A compensating advantage is that the diagonal 'fingerprint' provides a comparative picture of all the cystine peptides in one operation, whereas some cystine peptides are strongly adsorbed on ion-exchange resins and may be selectively overlooked in column techniques.

In this study of chymotrypsinogen A, no peptide

Fig. 8. Disulphide bridges of bovine chymotrypsinogen A. The half-cystine residues are shown by Roman numerals, and the broken lines indicate the peptic peptides numbered in Fig. 1 and Table 1. The sequence differs from that given by Hartley (1964) in positions 18, 19 and 215.

technique. We may therefore have some confidence in both the conclusions and the methods employed. Fig. 8 includes two corrections to the sequence of Hartley (1964) that are discussed in the Appendix to the present paper. The probable sequence of the cysteic acid peptides found in the peptic pH 6.5 diagonal (Fig. 1) is also indicated. We see that the I-IV bridge (CyS-1 to CyS-122) connects the A-chain of α -chymotrypsin (residues 1-13) to the B-chain (residues 16-146). The II-III bridge (CyS-42 to CyS-58) forms the 'histidine loop' that appears to be a common feature of pancreatic proteinases (Smillie & Hartley, 1964a) and is probably part of the catalytic site. The V-IX bridge (CyS-136 to CyS-201) connects the B-chain of α -chymotrypsin to the C-chain (residues 149-245) and together with the VII-X bridge (CyS-191 to CyS-220) forms the 'serine knot' that encloses the catalytic Ser-195. The VI-VII bridge (CyS-168 to CyS-182) forms the 'methionine loop' contained in the C-chain. The remarkable similarity of the 'histidine loop', the 'methionine loop' and the 'serine knot' in bovine chymotrypsinogen A, chymotrypsinogen B and trypsinogen and in porcine elastase has indicated that these four bridges may contribute to a common catalytic site in pancreatic proteinases (Hartley *et al.* 1965).

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