calculated as containing about 5 to 10 μg of polymyxin B was then spotted on to a plate at the starting point, together with similar samples of the standards. In addition to the fermentation liquor, mixtures of the fermentation liquor with several standard substances were also applied at the start line. Development was carried out as described in the preceding part. To ensure that no trace of solvent remained, the plates were dried at 80° for 1 h, then held in ammonia vapour for 5 min to neutralize any acetic acid possibly still present, since this would interfere with the biological testing on the agar plates inoculated with *Bordetella bronchiseptica*. A sheet of filter paper was

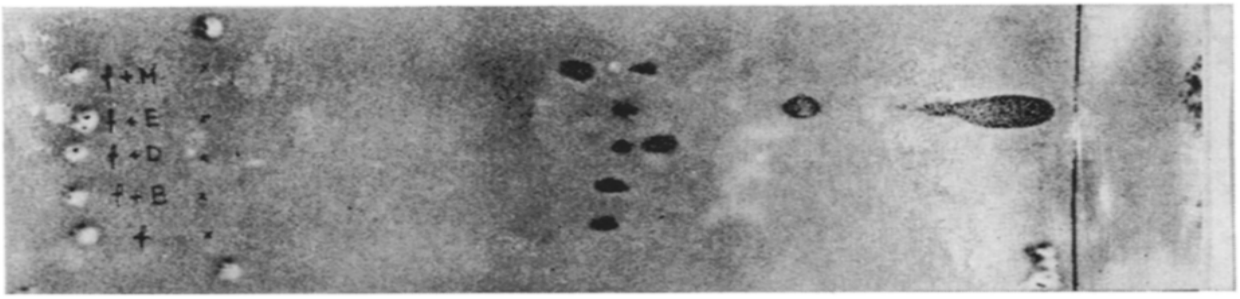

Fig. 3. Chromatography of fermentation broth containing polymyxin B, and of fermentation broths admixed with standard polymyxin substances. Spots made visible on an agar plate inoculated with *Bordetella bronchiseptica*.

laid on the agar plate and the chromatoplate was put on top. The latter and the filter paper were removed after 1 h and the agar plate was then incubated at 37° for 16 to 18 h. Fig. 3 shows that the active substance of the fermentation broths is distinct from all the standards, with the exception of polymyxin B. Thus the active substance produced is identical with it.

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Fingerprints of DNS-labeled protein digests on a millimicromole scale

The two-dimensional separation of protein hydrolysates on sheets of filter paper by combined electrophoresis and chromatography as developed by INGRAM[1] has become a standard technique for the study of similarities and differences among proteins. This so-called fingerprinting method has more recently been adapted to thin-

layer electrophoresis and chromatography by WIELAND *et al.*[2] In both cases the location of the fragments is normally detected with ninhydrin. The sensitivity of this reagent, which can vary considerably for different peptides, limits the minimum amount of protein digest required for a fingerprint to about 0.1 micromoles for separations on paper and to about 0.01 to 0.02 micromoles for the thin-layer method.

In this communication a new type of fingerprinting technique is described which requires as little as 1 to 2 millimicromoles of the enzymic hydrolysate of a protein. This has been achieved by labeling the peptides with the highly fluorescent DNS-residue according to GRAY AND HARTLEY[3]. This marker not only allows the detection of millimicromole quantities of peptides but also renders them more hydrophobic thereby facilitating their separation by two-dimensional chromatography on Kieselgel plates with organic solvent systems.

DNS-Cl* and chymotrypsin-free trypsin were purchased from Calbiochem, horse heart cytochrome c from Boehringer (Mannheim). Cytochrome c isolated from tuna fish hearts was a generous gift from Dr. D. M. BLOW (Cambridge, England). Both cytochromes were hydrolyzed with 5 % trypsin (w/w) at pH 8.5 and 37° for 2 h. The isolation of human γ-globulin and of a Bence-Jones protein, the reduction and alkylation of these proteins and the preparation of the L-chain of γ-globulin followed published procedures[4-6]. The reduced and alkylated globulins were dialyzed extensively against 0.01 *M* phosphate buffer (pH 8.2) and then digested with trypsin for 20 h at pH 8.2 and 37°.

The protein hydrolysates were incubated with a twenty-fold excess of DNS-Cl (w/w) at pH 8.0 overnight at room temperature. The reaction mixture, which contained appreciable amounts of DNS-OH, was applied to a Dowex 50-X8 column (0.2 × 2 cm) previously equilibrated with 0.01 *N* acetic acid (pH 3.5) (see ref. 3). The large excess of DNS-OH was eluted with the same medium. The labeled peptides could be displaced from the column with 1 *M* ammonia in 25 % acetone and were taken to dryness. Aliquots of this eluate were used for the separation on thin layer plates. The traces of DNS-OH and the DNS-NH_2 as well as some unknown side products still present in the peptide fraction served as convenient markers in the comparison of different fingerprints.

Thin layer plates were prepared with Kieselgel G (Merck) and dried for 1 h at 120°. The composition of the solvent mixtures used for the two-dimensional separation is given in the legends to the figures. Between the two runs, the plate was dried for 10 to 15 min at 100°. The labeled peptides could be detected on the wet plate by their intense yellow to orange fluorescence under a U.V. lamp and were marked on the rear side of the plate. DNS-OH and some unknown side products are easily discernible by their bluish fluorescence. Preliminary experiments have shown that the marked areas can be scratched off the plate and the peptides recovered by elution with 25 % dioxane or acetone.

Fig. 1a and b show a comparison of the DNS-labeled tryptic digests of horse and tuna fish cytochrome c. These fingerprints were obtained with 20 μg of hydrolysate, which is equivalent to about 1.5 millimicromoles of protein. From the known sequences of these proteins[7,8] and from earlier studies with the fingerprinting techni-

* Abbreviations: DNS-Cl = 1-Dimethylamino-naphthalene-5-sulphonyl chloride; DNS-OH = 1-dimethylamino-naphthalene-5-sulphonic acid; DNS-NH_2 = 1-dimethylamino-naphthalene-5-sulphonyl amide.

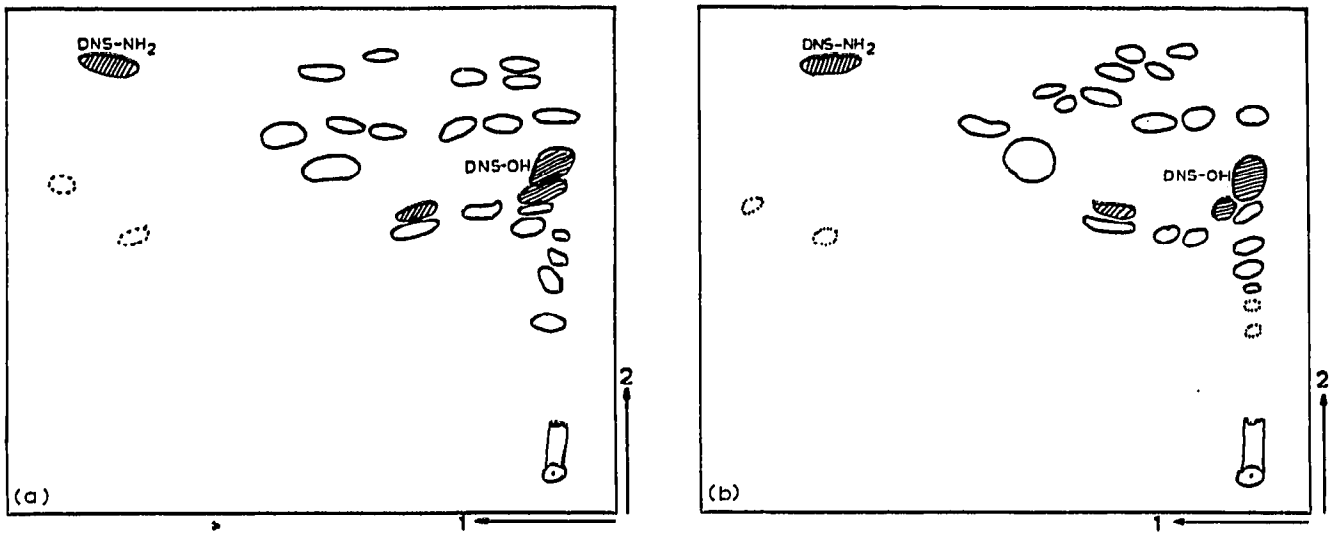

Fig. 1. Two-dimensional chromatography of DNS-labeled tryptic digests of (a) horse heart and (b) tuna fish heart cytochrome c. Solvent systems: (1) benzene–pyridine–acetic acid (80:20:5, v/v/v); (2) methyl acetate–isopropanol–conc. ammonia (45:35:20). The shaded spots represent DNS-OH, DNS-NH_2 and unknown side products.

que on paper[9], the number of major fragments to be expected is about 20 for horse and 18 for tuna fish cytochrome c, respectively. Due to incomplete hydrolysis of the clusters of basic amino acids occurring in these proteins, this number can vary with the time of the digestion. Minor components are therefore always observed. Two-dimensional chromatography on thin layer plates revealed 21 and 20 major and a few minor spots for horse and tuna fish cytochrome c, respectively. This is in good agreement with the expected numbers. The hemopeptides, barely visible as pink spots at these low concentrations, showed some tailing at the origin and could not be differentiated. Among the tryptic peptides six should be identical in both cytochromes[8]. On

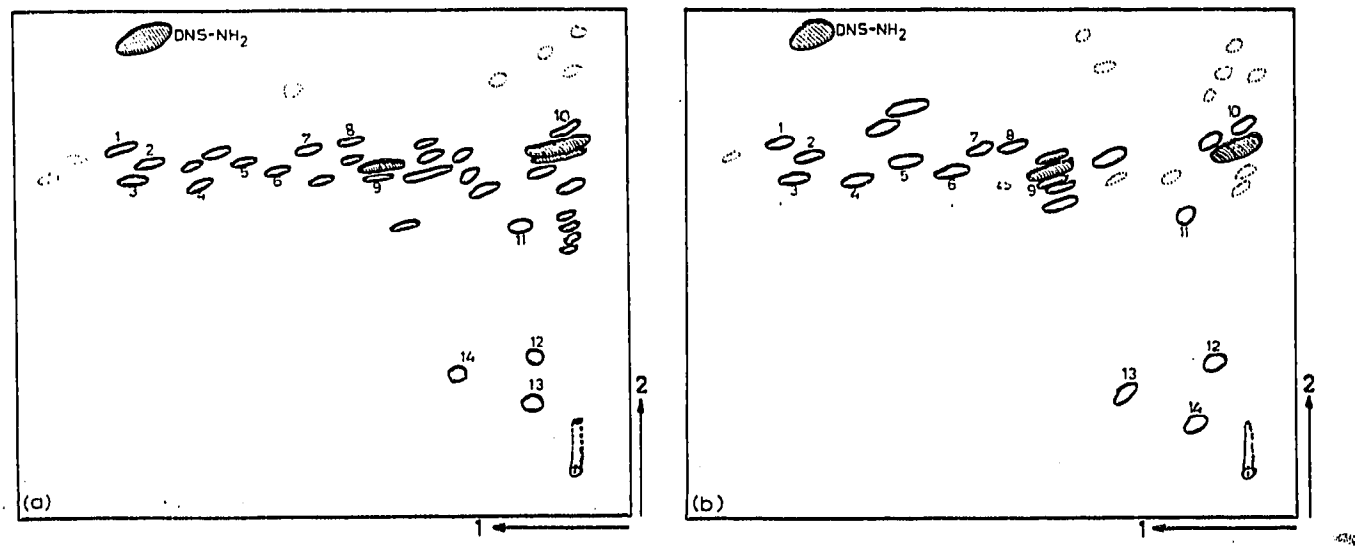

Fig. 2. Two-dimensional chromatography of DNS-labeled tryptic digests of (a) the L-chain of human γ-globulin, (b) a Bence-Jones protein. Same solvent systems as in Fig. 1. The spots occupying identical positions in both fingerprints were numbered 1–14.

the thin layer plates, according to our judgment, seven to nine spots were identical. Obviously, some of the differences are too small to be detectable by this method. It is, however, a general drawback of all fingerprinting techniques that only relatively drastic changes can be detected unambiguously and that the number of differences observed is generally smaller than the actual number.

A comparison of the tryptic digests of 20 μg (about one millimicromole) each of the L-chain of human γ-globulin and of a Bence-Jones protein is shown in Fig. 2a and b. Those peptides which upon close examination of a number of runs seemed identical were numbered 1–14. Some of the differences have to be attributed to the fact that the L-chain of normal γ-globulin is a mixture of serological types I and II. The Bence-Jones protein used in this study had N-terminal aspartic acid and is therefore presumably type I (ref. 10). After tryptic hydrolysis 21 major fragments could be detected on the thin layer plates. This is in good agreement with the investigations of HILSCHMANN AND CRAIG[11], who found 20 and 21 tryptic peptides for two other Bence-Jones proteins of type I.

In different experiments with a labeled hydrolysate the same overall picture was obtained. For a detailed comparison, however, only runs where both plates were prepared and dried simultaneously and all steps were done in parallel have been used.

This new fingerprinting technique requiring only millimicromole quantities of the enzymic hydrolysate of a protein is at least ten times more sensitive than previous methods. It should therefore facilitate the investigation of structural similarities and differences among proteins which bear an evolutionary, functional, serological or other relationship. It is the method of choice if only small amounts of a protein are available. Using this technique[12] it has already been possible to obtain fingerprints of blood clotting factors VII and X, where the preparation of larger amounts as required for other types of peptide separation would be extremely laborious.

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