PARTITION CHROMATOGRAPHY OF ENZYMIC DIGESTS OF INSULIN

by

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INTRODUCTION

Recent work by BUTLER, DODDS, PHILLIPS, AND STEPHEN[1] has shown that crystalline chymotrypsin breaks down crystalline insulin in a reproducible way, about 60% of the total nitrogen becoming soluble in 0.25 N trichloroacetic acid. This 'nonprotein' part was shown by diffusion experiments to have an average molecular weight of about 600, and this value is supported by the amino-nitrogen value of the peptide mixture, so that it consists of some 15 peptides, the average being a tetra or pentapeptide. The VAN SLYKE ninhydrin-CO_2 method[2] showed the presence of one or two free amino-acid residues in the mixture. The number of peptides is therefore not so great as to make their separation seem an impossible task. The methods of paper partition chromatography of CONSDEN, GORDON, AND MARTIN[3,4] have provided for the first time a means of separating minute amounts of amino-acids and small peptides in a precise and complete manner. The separation of di-and tri-peptides has been achieved using the partial acid hydrolysate from Gramicidin S (CONSDEN, GORDON, MARTIN, AND SYNGE[5]).

As far as the author is aware, no work has been published on the examination by this method of the peptides derived from enzymic breakdown of pure proteins.

This paper describes the application of these methods to the separation of the peptides derived from chymotryptic digests of insulin.

EXPERIMENTAL

The peptide mixtures used were prepared from insulin in the following way. Crystalline insulin (Boots) at a concentration of 5 mg per ml, was digested for 19 to 24 hours at 25° C in p_H 8.3 phosphate buffer with $3.2 \cdot 10^{-4}$ (Trypsin units) of chymotrypsin per ml of digest. The chymotrypsin was prepared by the activation of eight times recrystallized chymotrypsinogen with a very little crystalline trypsin.

The insulin, trypsin and chymotrypsinogen were obtained from beef pancreas. These digestion conditions always produced 50 to 65% of nitrogen soluble in 0.25 N trichloroacetic acid. The whole digest was precipitated by making it up to 0.25 N with trichloroacetic acid by adding one-third of its volume of 1 N trichloroacetic acid, and the precipitate filtered off. The filtrate was then extracted continuously with ether.

Some hydrochloric acid was added towards the end of the extraction to assist the removal of the trichloroacetic acid. The whole extraction occupied about 48 hours and about 95% of the acid was removed, as determined by titration of the extract. The ether

References p. 347.

extract, provided entrainment is avoided, contains less than 0.3% of the non-protein nitrogen. The solution of peptides was then neutralized to p_H 7.5 and evaporated down partly below 40° C *in vacuo* and mainly at room temperature *in vacuo* over desiccants, to avoid any undesirable effects of prolonged heating. In all the digests prepared a small precipitate appeared during the ether extractions, being not less than 0.6% of the insulin taken, the upper limit (1-2%) being difficult to assess as the precipitate adhered as a tenacious film to the sides of the extraction apparatus. A similar precipitate was observed when a digest was only 4 times extracted in separating funnels. For the partition chromatography, Whatman No. 1 paper was used throughout. The peptide material was applied in aqueous solution between two pencil lines 0.5 cm apart drawn some 7 cm from one edge of the sheet. About 10 mg peptides was applied along a 50 cm line in this way. The apparatus used was similar to that described by CONSDEN, GORDON, AND MARTIN[3]. Troughs made of polyethylene ('polythene') tubing or from aluminium sheet were found to be suitable and durable. After the run the sheets were dried over an electric hotplate in a good draught. The sheets were then examined in ultraviolet light, (see PHILLIPS[6]) using the light from a "Hanovia" ultraviolet lamp screened by a 1 cm thick Wood's glass filter in a dark room. Thorough drying of the paper beforehand is essential. The fluorescence revealed was generally very faint, and of little use in fixing the position of an amino-acid or peptide spot or band unless the quantity on the paper was large (*e.g.*, 5 μg per sq. cm) compared with the amount required to give a ninhydrin colour reaction. However, the method shows up the vagaries of flow of the solvent down a sheet, so that if a one-dimensional run is done with a continuous line or a long row of separate spots of material at the origin, it is possible, developing the edge strips only with ninhydrin, to cut out the bands with some accuracy. The method has also shown that there are highly fluorescent substances in some of the solvents used which travel on two-dimensional chromatograms as spots as small as those obtained with amino-acids. The scope of the paper partition method could thus be extended to further types of substances.

After the ultraviolet examination, the sheet or samples of it are sprayed (avoiding drenching the paper), using an insecticide spray with 0.1% ninhydrin in water-saturated butanol containing 1% of pyridine (see HARDING AND MACLEAN[7]). With the peptide chromatogram considerable heating was required to bring up the colour at all, much of the colour was yellow, and background colour increased rapidly on strongly heating the paper so that this reaction is far less effective here than with amino acids. Using a range of quantities of starting material, the larger amounts, after chromatography and development with ninhydrin not only show increased colour intensities but reveal extra bands as well. One must therefore compromise between taking too much starting material, which will overload the chromatogram, or too little material, which will not be revealed by the rather insensitive peptide ninhydrin-reaction. In some cases the colour development continues or alters in hue long after heating with ninhydrin. Grey colours especially tend to become purple. Such colour changes of peptides with ninhydrin have been noted already by CONSDEN, GORDON, AND MARTIN[4].

RESULTS

A. One-dimensional chromatograms of the peptide mixture

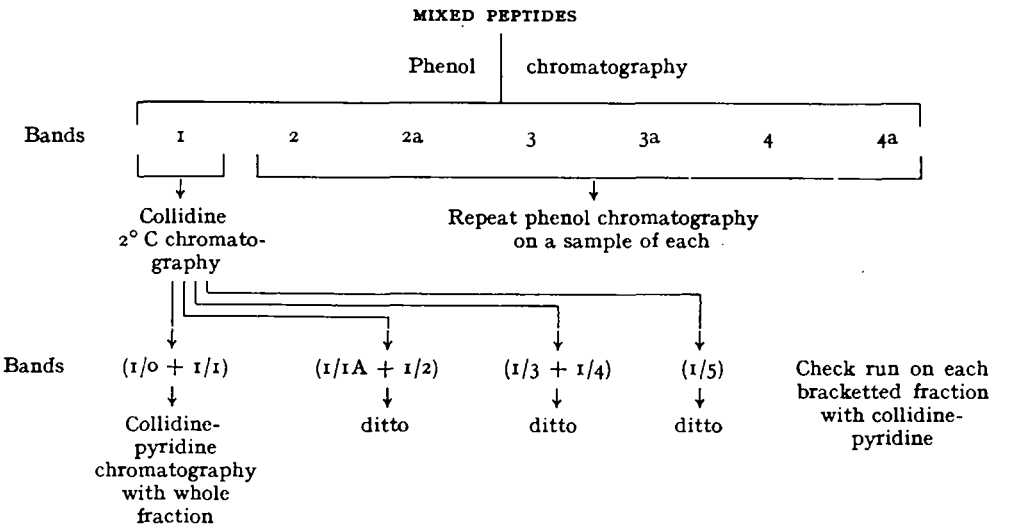
Table I gives the scheme of separation applied to the peptide mixture. For the

References p. 347.

first phenol run about 1 g of peptides was chromatographed (about 10 mg per sheet) and edge strips cut for testing. With ninhydrin, four bands appeared: (1) R_F 0.0 to 0.20, (2) 0.25 to 0.50, (3) 0.60 to 0.70, (4) 0.85 to 0.95. The positions and distinctness varied and the gaps were numbered 2a, 3a, 4a. After correlating the sheets the bands were cut out, eluted with water and the eluates evaporated to dryness *in vacuo* below 50° C. Phenol band 1 was then chromatographed with collidine. This was done at 2° C in a refrigerator, to increase the water content and peptide solvent power of the collidine. Ninhydrin treatment of samples of these sheets showed seven bands, (1/0, 1/1, etc.), but some were too close to cut out separately and were eluted in pairs (see Table I). Check runs on samples of these fractions, (1/0 + 1/1), etc., were then made with a collidine-pyridine-water solvent (47:15:38 by volume) which showed that they were complex, some of the components being common to all fractions. As the resolution by collidine-pyridine in the check run seemed to be better than in the bulk collidine run prior to it, the whole of each bracketted fraction (1/0 + 1/1); (1/1A + 1/2); (1/3 + 1/4); (1/5), was run with collidine-pyridine. With ninhydrin they now all gave a similar pattern, differing only in the relative proportions of the bands. The corresponding bands from each were collected together and eluted. Resolution was still incomplete however, as check runs on samples of these eluates showed traces of other bands. Collidine or collidine-pyridine chromatography therefore, can only produce a partial resolution of the mixture.

TABLE I

SCHEME OF CHROMATOGRAPHY OF THE PEPTIDE MIXTURE FROM INSULIN



When the other phenol bands and gaps, 2, 2a, etc., were checked by running samples of them again with phenol, the same effect was revealed. Band 2 for instance showed the presence of bands 1 and 3 material, and even the 'gap' 2a contained material from bands 1, 2 and 3.

Strip chromatograms were also run using the peptide mixture from several different chymotryptic digests of insulin. These all gave a similar band pattern with phenol, indicating at least a general reproducibility of the enzyme action.

References p. 347.

B. Chromatography with solvents other than phenol

Other solvents were tried after the main work described above. Cyclohexane, benzene, ethyl acetate and n-amyl alcohol did not move the material from the application point. Benzyl alcohol and aniline pyridine (1 : 1) gave only 3–6 cm movement in 18 hours. All solvents were used saturated with water. With n-butanol, movement was slow. A 40 hour run with digest C8F produced the following sequence: (1) R_F 0.0 to 0.05, purple and violet ninhydrin colours; (2) 0.09, pale violet; (3) 0.16, grey-purple; (4) 0.22, pale violet; (5) 0.32, pale green; (6) 0.37, pale purple. Band (1) showed three colour changes so that there is evidence of 8 substances here, and the faster materials formed good spots.

n-Butanol saturated with an equal volume of five times diluted glacial acetic acid gave the best results obtained so far, and with the same peptide mixture C8F gave the following one-dimensional chromatogram; (1) R_F value 0.01, pale violet (V) ninhydrin colour; (2) 0.03, pale purple (P); 0.04, yellow; (4) 0.05, blue-grey; (5) 0.07, P; (6) 0.10, pale P; (7) 0.12, pale red; (8) 0.15, pale V; (9) 0.16, pale red; (10) 0.20, pale P. These bands formed a continuous streak but the ninhydrin colour changes were quite distinct. Then followed as separate spots: (11) 0.32, red; (12) 0.52, grey; (13) 0.61, Pale P; (14) 0.73, pale P.

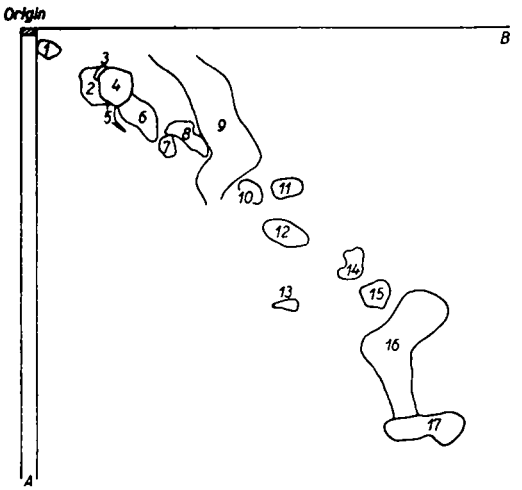


Fig. 1. Ninhydrin colours of the spots as numbered: 1. pale grey; 2. pale blue; 3. blue; 4. blue; 5. violet (V); 6. pale V; 7. very pale V; 8. strong V; 9. V; 10. pale V; 11. pale V; 12. very pale V; 13. pale V; 14. pale grey; 15. pale V; 16. purple; 17. pale purple; 0.5 mg peptide mixture applied at ORIGIN, run in direction A then in direction B with n-butanol-acetic acid.

Sometimes the fastest spots gave a green ninhydrin colour. The poor resolution seen in the phenol chromatograms was shown to be largely absent from the butanol-acetic acid chromatograms by running a two-dimensional sheet using butanol-acetic acid as solvent in both directions. Had overlapping occurred in the first run, the subsequent movement in the other direction would have destroyed the spot pattern and revealed streaks of colour with ninhydrin. Fig. 1 shows a copy of the results with digest C8F, in which 17 spots appeared, with evidence of spread only in spots 9 and 16. The colours on the original faded very rapidly. Two-dimensional chromatograms with phenol or collidine in both directions or with phenol and then collidine were unsuccessful. Ninhydrin revealed only large vague areas of colour.

It is intended to continue the separation with butanol-acetic acid using longer sheets of paper to increase the resolution, and to characterize the individual peptides obtained.

DISCUSSION

The phenomena described in the experimental part of this paper suggest that with phenol as the chromatographic solvent, several at least of the peptides obtained by the chymotryptic digestion of insulin (averaging tetra-penta-peptides), are spaced out over

References p. 347.

a large part of the distance traversed by the solvent. As a result, material eluted from cross-cuts from different parts of a one-dimensional run show identical bands on repeating the chromatography. Collidine and collidine-pyridine as solvents may show similar effects, and it was this difficulty that made the separation procedure both complicated and unsuccessful. No definite cause of the failure of phenol or of the improvement of separation by the subsequent use of n-butanol-acetic acid as solvent is apparent though some of the factors involved have been considered.

1. In the first place, the ninhydrin reaction results must be treated with reserve. It would seem that peptides of the size of pentapeptides can form good spots on suitable paper chromatograms and that the formation of broad bands of ninhydrin colour should be taken as evidence of an unsuitable system. The finding that phenylalanine gives a green colour with this reagent when slight heating has been used to dry the chromatograms may have special significance in the case of the peptide mixture used here. The specificity of chymotrypsin according to BERGMANN AND FRUTON[8] requires a phenylalanyl or tyrosyl residue on the carboxyl side of the peptide bond split. The aromatic residue may have its amino group free, but the presence of an α-carboxyl group in the immediate vicinity inhibits the action of the enzyme. The insulin submolecule of molecular weight 12000 contains 9 tyrosine and 6 phenyl-alanine residues according to CHIBNALL[9], whilst BRAND[10] finds 8 tyrosine and 6 phenylalanine residues per 12000 weight. Only the positions of two of these are known, the terminal phenylalanyl residues as found qualitatively by JENSEN AND EVANS[11] and confirmed quantitatively by SANGER[12]. BUTLER *et al.*[1] found that for digests of insulin producing 66% non-protein nitrogen, 15–16 peptide bonds per unit of molecular wt 12000 were broken. The accuracy is not high in this measurement, but the agreement between the number of bonds broken and number of aromatic amino acids contained in the submolecule of 12000 may well be significant. This is supported by the finding that the precipitable 'core' of insulin (weight 5000), remaining after digestion, contains 5 aromatic amino-acid residues — 4 tyrosine and 1 phenylalanine, whilst the soluble peptide fraction (total combined weight about 7000) contains 10, 5 tyrosine and 5 phenylalanine (PHILLIPS[13]).

This may mean that after digestion, four aromatic acid residues are at the ends of the four polypeptide chains of the 'core' and that of the 15 or so peptides in the trichloroacetic acid filtrate, 10 have aromatic amino-acids at their carboxyl ends, half of which would then be phenyl-alanyl residues. It is also known that approximately 2 free amino-acid molecules are produced per submolecule of insulin during the chymotryptic digestion. The enzyme specificity (FRUTON AND BERGMANN[14]) makes it likely that the two phenyl-alanine residues with their amino-groups free at the ends of two of the chains of the submolecule, will be split off as free amino acids. The experiment described, using a range of quantities of the peptides with n-butanol-acetic acid as chromatographic solvent, yielded several spots with green ninhydrin coloration, which may therefore be indicative of those peptides containing phenylalanine.

2. The conditions under which substances may spread on partition chromatograms have been studied especially by LUGG AND OVERELL[15]. These authors, studying acids such as malic, citric and tartaric, concluded that 'tailing' (the formation of an extended tail behind the main spot) is due to a change in partition coefficient in favour of the aqueous stationary phase as dilution of the material proceeds during the movement, and that in their case this change was due to change in the degree of ionisation. In the

References p. 347.

work described here the phenol saturated with water used for the peptide chromatography had a p_H of 5.4 (glass electrode), which lies within the zwitterionic range of all amino-acids and usual peptides. In n-butanol-acetic acid (p_H 2.8) the α, β and γ COOH groups will become far less ionised, favouring non-aqueous phase solubility.

3. The effect of overloading a chromatogram with solutes might have produced the spreading effects seen. With amino acids 'tailing' is rarely seen. The effect of using 80 μg of one acid compared with using only 5 μg is to increase the dimensions of the spot in a uniform way both in the direction of solvent flow and at right angles to it. This characteristic has recently been made the basis of the quantitative measurement of the amino acid in a spot (FISHER[16]). I have found that phenol-ammonia does occasionally produce a gross elongation of an amino acid spot, especially of aspartic and glutamic acids when the quantity present is high (60–100 μg). The effect is eliminated if the run with *e.g.*, butanol-acetic acid, is done before the phenol run. (It is noteworthy too that glycine and serine are separated better when the solvents are used in this order). With the peptide mixture, using a range of quantities at the starting point varying from 28 to 475 μg/cm length of starting line, and phenol as solvent, a similar pattern was produced in all cases, at the highest concentration the bands spreading out and almost merging, while for quantities below 100 μg/cm, the faster bands were absent from the developed chromatogram, the ninhydrin reaction apparently being too insensitive. The quantities used in the main chromatography described in this paper were about 200 μg/cm. The suitability of the solvent depends partly on the time available to dissolve the solute from the starting-line. This time is increased for instance if the solvent is relatively viscous or the paper inclined so that the solvent speed is reduced or the material placed further down the paper away from the solvent, or by the use of a paper of finer texture. If the critical time is exceeded, the origin becomes equivalent to several separated origins and a streak is bound to result. In practice the time available is very short, for the commonly used solvents are not at all viscous and at the top of the paper at the beginning of a run the solvent moves more rapidly over the paper than at any other time during the development. Thus, for example, on No. 1 Whatman filter paper, phenol and butanol-acetic acid (both solvents saturated with water) took 75 sec and 65 sec respectively to move over a 1 cm wide spot on a level with, and 1 cm from the solvent reservoir, and 3¾ min and 3 min respectively to move the next 1 cm. Hence if phenol fails as a solvent on this account, n-butanol-acetic acid must have a much more rapid solvent action.

A complication arises in the case described here, since buffers are used in the digestion solution which subsequently go into the trichloroacetic acid filtrate and are chromatographed with the peptides. The proportion of these salts is about 2 mg mainly as phosphate, per 1 mg peptides. One effect of this salt can readily be seen if the solid is shaken up with water-saturated phenol or butanol, when an aqueous salt layer quickly forms. This means that a strong salt solution will collect in the upper part of the paper and cause undersaturation of the solvent passing over it, though the ions of the salts can move down the chromatograms as has been shown by WESTALL[17].

4. Adsorption does not seem to play a great part in determining the flow of ordinary amino-acids and peptides on paper (CONSDEN *et al.*[3]). It would seem that an adsorption effect can be exhibited with suitable solutes, *e.g.*, N–2:4 dinitrophenyl (DNP) amino-acids. If these acids are run on paper in the usual way but using water or weak aqueous buffers only as solvents, the different DNP-amino acids run as spots at different speeds.

References p. 347.

With free amino-acids, or the peptide mixture from insulin under these conditions, all the material runs fast, either at the front or nearly so.

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SUMMARY

The methods of paper partition chromatography have been applied to the separation of the trichloroacetic-acid-soluble peptides from chymotryptic digests of insulin. With phenol and collidine as solvents many of the peptides spread out and could not be separated, but n-butanol-acetic acid as solvent produced several well-defined spots which gave characteristic ninhydrin colours. The significance of these colours with regard to enzyme specificity is discussed and also some of the factors influencing the movement of the peptides on the chromatograms.

RÉSUMÉ

Les méthodes de la chromatographie de CONSDEN *et al.*[3] sur le papier ont été appliquées à la séparation des peptides de l'insuline (obtenues par l'action du ferment chymotrypsine) qui sont solubles dans l'acide trichloroacétique. Avec le phénol et la collidine comme solvants, plusieurs des peptides se sont répandues sur le papier, et l'on ne peut pas les séparer. Mais le mélange n-butanol-acide acétique comme solvant a produit plusieurs taches bien définies qui ont donné des couleurs caractéristiques avec la ninhydrine. L'importance de ces couleurs à l'égard de la spécificité du ferment et aussi de quelques agents qui influent sur le mouvement des peptides sur le papier est discutée.

ZUSAMMENFASSUNG

Die Methoden der Chromatographie an Papier von CONSDEN und Mitarbeitern wurden zur Trennung von in Trichloressigsäure löslichen Peptiden aus Insulin, das mit Chymotrypsin verdaut war, angewendet. Mit Phenol und Kollidin als Lösungsmittel, breiten sich viele Peptide weit aus und können nicht getrennt werden; dagegen erhält man mit n-Butanol-Essigsäure mehrere gut definierte Flecke, die mit Ninhydrin charakteristische Färbungen geben. Die Bedeutung dieser Färbungen in Bezug auf die Spezifizität des Fermentes, sowie einige, die Verteilung der Peptide auf dem Chromatogramm beeinflussende Faktoren, werden erörtert.

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